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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/00, 9/00, 38/19, 48/00, 38/20, 39/21, C12N 15/19, 15/62, A61K 31/70, C12N 15/48

A2

(11) International Publication Number: **WO 99/16466**

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/US98/20321

(22) International Filing Date: 29 September 1998 (29.09.98)

(30) Priority Data:

60/060,338	29 September 1997 (29.09.97)	US
08/990,180	12 December 1997 (12.12.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/990,180 (CIP)
Filed on	12 December 1997 (12.12.97)
US	60/060,338 (CIP)
Filed on	29 September 1997 (29.09.97)

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: VACCINE COMPOSITIONS AND METHODS OF ENHANCING VACCINE EFFICACY

(57) Abstract

The invention provides methods, vaccine compositions and plasmid constructs which enhance the immune response of a vaccine.

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VACCINE COMPOSITIONS AND METHODS OF ENHANCING VACCINE EFFICACY

RELATED APPLICATION(S)

This Application is a continuation-in-part of U.S. Application 08/990,180 filed December 12, 1997 and claims the benefit of U.S. Provisional Application 60/060,338, filed on September 29, 1997, the teachings of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Vaccination induces specific immunity in a host against foreign viruses, bacteria or parasites. Many types of infectious agents and their products have been used as vaccines. Some examples include attenuated and inactivated bacteria (e.g., Bordeltella pertussis), purified antigens (e.g., a Hemophilus influenza protein), synthetic antigen vaccines (e.g., synthetic peptides), and most recently, DNA vaccines (e.g., DNA encoding a desired antigen).

However, not all vaccine compositions are capable of inducing, or eliciting, an immune response sufficient to protect the host against the challenge of infection. Therefore, there exists a need to enhance the immune response for vaccines that fail to elicit a protective immune response in a host.

SUMMARY OF THE INVENTION

The present invention relates to vaccine compositions and methods to enhance one or more parameters of an immune response to a vaccine by using cytokine constructs. The vaccine compositions and methods of the present invention are based on the discovery that administration of a cytokine or cytokine fusion protein subsequent to administration of a vaccine enhances the immune response.

Specifically, the invention encompasses vaccine compositions which include the vaccine and a cytokine, cytokine fusion protein, or plasmid that is

encased within a timed-release drug delivery formulation. The vaccine compositions can be administered to a host at a single timepoint, e.g. both the vaccine and timed-released cytokine/Ig formulation are at the same time. The vaccine component and the cytokine component can be administered admixed in a single dose (e.g. an effective amount of the two components in solutions) to the host, or, alternatively, can be administered to the host separately, but substantially simultaneously. The fusion protein is a cytokine/Ig fusion protein and the protein delivery formulation can release the fusion protein subsequent to administration of the vaccine. More specifically, the timed-release formulation can release the fusion protein or plasmid gradually beginning substantially immediately after vaccine administration until 7 days after the vaccine administration. Release of the cytokine/Ig fusion protein subsequent to administration of the vaccine significantly enhances the response of the immune system as measured by the immunologic parameters described herein. Alternatively, the cytokine/Ig can be administered within a few days subsequent to administration of the vaccine, as described herein.

In particular, the claimed invention encompasses a mammalian cytokine/Ig fusion protein, wherein the cytokine is IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-15, TNF-α , TNF-β, IFN-γ, or GM-CSF. An embodiment of the claimed invention is a vaccine composition comprising the murine nucleic acid sequence (SEQ ID NO: 3) or amino acid sequence (SEQ ID NO: 4), or the human cytokine fusion nucleic acid (SEQ ID NO:1) or amino acid sequence (SEQ ID NO: 2). Specifically encompassed is a vaccine composition comprising an amino acid sequence encoded by SEQ ID NO: 1 or 3, or the amino acid sequence of SEQ ID NO: 2 or 4. The vaccine composition can also comprise a nucleic acid sequence comprising SEQ ID NO: 1 or 3, a nucleic acid that encodes SEQ ID NO: 2 or 4, a nucleic acid that hybridizes to SEQ ID NO: 1 or 3, the complement thereof, or RNA that is transcribed therefrom.

The invention further relates to methods of enhancing vaccine immunogenicity. As described herein, the present invention encompass enhancing the immune response elicited by a vaccine comprising administering a cytokine or a cytokine fusion protein to the host subsequent to vaccination. Alternatively, the

present invention also encompasses methods of enhancing the immune response elicited by a vaccine comprising administering a vaccine composition to the host comprising a vaccine and a timed-release cytokine or cytokine fusion protein. Vaccines amenable to enhancement by the methods described herein include DNA vaccines as well as conventional vaccines. Such vaccines can be useful to treat or protect hosts affected with a variety of diseases or syndromes including AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, cancer and influenza. If the cytokine/Ig fusion protein is administered subsequent to the vaccine administration, it can be administered as a soluble protein or as a DNA sequence encoding the protein. The immunological enhancement observed with these compositions and methods described herein is manifested by enhancement of one or more immunologic parameters such as an antibody response, a cellular proliferative response as well as cytotoxic T-lymphocyte levels.

As described herein, a period of maximal efficacy for the use of cytokines and cytokine fusion proteins as adjuvants to vaccines has now been identified. The cytokine constructs are maximally effective if delivered in a window of time lasting several days following vaccination. This is the first demonstration that a cytokine or cytokine/Ig fusion construct administered subsequent to administration of a vaccine can significantly enhance the immunogenicity of a vaccine.

The invention further relates to method for administering IL-2 therapy or for treating patients having cancer, an immunodeficiency related diseases, or infectious diseases by administering a IL-2/Ig fusion protein. The IL-2/Ig fusion protein has a longer half life than does IL-2, and therefore, warrants administration less frequently than does IL-2. Accordingly, IL-2/Ig administration induces less negative side effects than does IL-2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the immunogenicity of dicistronic pV1J-gp120/cytokine vaccines compared with the control pV1J-gp120 vaccine. Geometric mean titers with standard errors of total serum anti-gp120 antibodies are shown.

Figure 2A is a graph showing the comparison between the administration of the vaccine with and without the administration of the IL-2 protein.

Figure 2B is a graph showing the differences between the IL-2/Ig protein and the IL-2 protein. For both experiments, geometric mean titers (GMT) with standard errors of total serum anti-gp120 antibodies are shown. GMT of sham injected mice were <10.

Figure 3 is a graph showing the soluble IL-2/Ig protein administered systemically enhances the splenocyte proliferative response to the DNA vaccine pV1J-gp120. Results shown are means involving 4 mice per group each assayed in triplicate.

Figure 4 is a graph depicting the effect of the soluble IL-2/Ig protein on the CTL response. Results shown are means from 4 separate animals each assayed in duplicate. Circles represent the mice that received pV1J-gp120 plus systemic IL-2/Ig treatment, squares represent the mice that received pV1J-gp120 plus the control Ig treatment, and triangles represent the mice that received only the sham pV1J plasmid.

Figures 5A-C are graphs that illustrate the immunologic effects of administering plasmid IL-2/Ig at different times. Results shown are means from 6 separate animals each assayed in duplicate. Circles represent the mice that received pV1J-gp120 plus pV1J-IL-2/Ig on Day +2, triangles represent the mice that received pV1J-gp120 plus pV1J-IL-2/Ig on Day -5, and squares represent the mice that received only pV1J-gp120.

Figures 6A-B are graphs that demonstrate the results from administration of pV1J-IL-2 or pV1J sham plasmid. Both of these plasmids fail to augment significantly the humoral (Figure 6A) and CTL response (Figure 6B) to the DNA vaccine pV1J-gp120. Results shown are means involving 6 individual animals each assayed in duplicate. Circles represent the mice that received pV1J-gp120 plus pV1J-IL-2 on Day +2, and squares represent the mice that received only pV1J-gp120.

Figures 7A-B are graphs that show the effect of cytokine timing on the humoral immune response to the DNA vaccine pV1J-gp120. In Figure 7A, groups

of Balb/c mice (N=6) were immunized i.m. with 50 μg pV1J-gp120 on Day 0 plus 200 μg pV1J-GM-CSF on Day -5, 0, +2, or +5. In Figure 7B, groups of C3H mice (N=6) were immunized i.m. with 50 μg pV1J-gp120 on Day 0 plus 200 μg pV1J-IL-2/Ig on Day -5, 0, +2, or +5. In both experiments, mice were bled after 4 weeks, and sera were tested for specific anti-gp120 antibodies by ELISA. Geometric mean titers with standard errors of total serum anti-gp120 antibodies are shown.

Figure 8 illustrates the plasmid which was modified to make a plasmid that encodes the IL/Ig fusion protein.

Figures 9A-B depict the DNA and amino acid sequence for coding region of the murine IL-2/Ig fusion protein. The nucleic acid sequence for the murine IL-2/Ig fusion protein is SEQ ID NO: 3, and the encoded amino acid sequence is SEQ ID NO:4.

Figures 10 A-E show the nucleic acid sequence (SEQ ID NO: 1) that encodes the human IL-2/Ig fusion protein and the corresponding amino acid sequence (SEQ ID NO: 2).

DETAILED DESCRIPTION

A cytokine is a protein which mediates cell to cell communication in the immune system as well as assists in regulating the development of the immune system. Cytokines serve as cell communicators because they have the ability to exhibit various types of action including autocrine (binding to the same cell that secreted it), paracrine action (binding to the nearby cell) and sometimes also endocrine action (binding to a distant cell). Kurby, James, "Immunology," W.H. Freeman and Co., p247 (1992). This cell to cell communication allows a cytokine to regulate the intensity and duration of the immune response by stimulating or inhibiting the proliferation of various cells or their secretions.

Several types of cytokines exist. Some examples are IL-1 (IL stands for Interleukin), IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-15, TNF-α (TNF stands for Tumor Necrosis factor), TNF-β, IFN-γ (interferon-γ), GM-CSF. Many others exist, some of which have not yet been identified. As defined herein,

the term "cytokine" refers to all cytokines including those which have not yet been discovered or identified. The present invention encompasses these listed cytokines, other known cytokines, and those cytokines which have not yet been identified. As described herein, the cytokines of the present invention are modified to increase the length of time the cytokine circulates in a host and by making the cytokine a divalent ligand. This length of time is scientifically measured by the cytokine's half-life, the amount of time required for one-half of an amount of the cytokine to be lost through biological processes. The half-life is significantly increased by fusing, or linking, the cytokine to all, or a portion of, an immunoglobulin or Ig antibody protein, thereby producing a cytokine/Ig fusion protein.

The cytokine fusion protein can be made from proteins derived from various species (e.g., mammals, human, non-human primate, murine, etc.). For example, a human cytokine fusion protein is defined as a cytokine fusion protein derived from the human cytokine homolog (e.g., IL-2). Similarly, a murine cytokine fusion protein is defined as a cytokine fusion protein derived from the murine cytokine homolog (e.g., IL-2).

In the cytokine fusion proteins of the present invention, the cytokine is typically linked to the Ig protein at the Fc region of the Ig molecule. All or a portion of the immunoglobulin molecule can be used and any immunoglobulin molecule can be used, for example IgG or IgA. As defined here, "fusion protein" or "cytokine/Ig," refer to the cytokine fusion protein. The terms that include the actual cytokine such as IL-2/Ig or IFN-γ/Ig refers to the corresponding cytokine fusion protein, i.e. that IL-2 protein or the IFN-γ protein fused with an Ig antibody polypeptide. Methods of making cytokine fusion proteins are known in the art. Larochelle, *et al.*, "Fusion Proteins that Include Antibody and Non-antibody Portions," PCT Application No. PCT/US95/00974, filed February 1, 1995, the teachings of which are incorporated by reference in their entirety; Strom *et al.*, "Chimeric Cytokines and Uses thereof," Serial No. 08/431,535, filed April 28, 1995, and the parent application, Serial No. 08/355,502, filed December 12, 1994, the teachings of both are incorporated by reference in their entirety;

Sytkowski, A. *et al*, "Production and use of recombinant erythropoietin/Immunoglobulin fusion proteins," Serial Number 08/889,829, filed on July 10, 1997, the teachings of which are incorporated by reference in their entirety.

As described herein, a plasmid can produce a functional cytokine fusion protein (i.e., a cytokine fusion protein having the activity of the native or unfused cytokine). A plasmid is an independent self-replicating DNA molecule that carries genes which encode a protein. The invention utilizes a plasmid which encodes a cytokine fusion protein. Methods of constructing a plasmid which encode a cytokine fusion protein are described herein. As used herein, the term "cytokine fusion protein" refers to the protein which is produced using methods known in the art, including those incorporated by reference as well as the plasmid which encodes the protein. Therefore, cytokine fusion protein, fusion protein, or fusion protein administration or any permutation thereof means administering the cytokine fusion protein or a plasmid which encodes the cytokine fusion protein.

A vaccine is any composition intended for active immunological prophylaxis or a composition which elicits a protective immune response. Several types of vaccines are known, such as live organism attenuated vaccines, killed organism vaccines, subunit vaccines, recombinant or engineered protein vaccines, toxoid vaccines, peptide vaccines, live vector vaccines, and DNA vaccines. For example, a live organism attenuated vaccine is a vaccine which modifies the organism, bacteria or virus to illicit an immune response without causing disease. In another example, a killed organism vaccine is a vaccine in which a non-living organism, bacteria or virus elicits an immune response without causing disease. The vaccine composition utilizes a specific portion of the organism in one form or another to illicit a protective immune response. The invention embodies these vaccines, conventional vaccines, experimental or research vaccines, other known vaccines, as well as those developed in the future. A vaccine can also modulate the immune response for therapeutic purposes.

A "potent vaccine" refers to a vaccine which is capable of eliciting a protective immune response in the majority of the patient population. For

example, the vaccine used in Example 1, the gp120 DNA vaccine for the HIV virus induces seroconversion or T-cell activity in >90% of inoculated mice in the absence of cytokine augmentation and is considered a potent vaccine. A potent vaccine that induces seroconversion or T-cell activity in >50%, including ranges that are >60%, >70%, >80% or >90% are also considered a potent vaccine. A "sub-optimal vaccine" is a vaccine with a seroconversion of <50%, including ranges that are <40%, <30%, <20% or <10%. Suboptimal vaccines are also those vaccines which produce less than desired efficacy.

As used herein, the term "disease" refers to an interruption, cessation, or disorder of bodily functions, systems, or organs for which a vaccine is or could potentially be effective. The invention embodies all diseases that affect vertebrates in which an vaccine could be useful including those caused directly or indirectly by an organism or pathogen as well as toxins derived therefrom. Vaccines for some disease are either currently ineffective or are sub-optimally effective. Examples of diseases which affect vertebrates, and in particular, mammals, including humans, are AIDS, malaria, tuberculosis, Hepatitis B, Hepatitis C, cancer and influenza. Methods and compositions of enhancing the immunogenicity of a vaccine for these particular diseases are encompassed by the present invention.

A timed-release drug delivery formulation or device refers to a formulation or device which is capable of releasing a substance, such as a fusion protein, into a host at or after one or more temporal points. This formulation also refers to the ability to release the substance at a predetermined temporal point in a bolus fashion or as a gradual release. Various examples of this formulation include microspheres, liposomes, microcapsules, implants, non-degradable materials, biodegradable material, and other polymers which are used for controlled temporal release. Methods for timed release delivery formulations involving cytokine release in conjunction with vaccine development are known in the art. Zhao, Z. *et al.*, "Controlled Delivery of Antigens and Adjuvants in Vaccine Development," *J. of Pharmaceutical Sciences*, Vol. 85, No. 12, 1261-1270, December 1996. Furthermore, other known methods for timed release delivery formulations used for biologically active agents also can be used for cytokine-Ig fusion proteins or

plasmids. Berstein *et al*, "Modulated release from Biocompatible Polymers," Patent No. 5,565,297, issue date, August 12, 1997, the teachings of which are incorporated by reference in their entirety; Okada, H. *et al.*, "Biodegradable microspheres in drug delivery," *Critical Review in Therapeutic Drug Carrier Systems*, 12(1):1-99 (1995); Weiner, A., *et al.*, "Liposomes for protein delivery: selecting manufacture and development processes," *Immunomethods,* 4(3):201-9 (Jun 1994); Blanford, A., *et al.*, "Review: implants," *J. of Ocular Pharmacology*, 10(4): 691-701, (Winter 1994); Gombotz, et al., "Very Low Temperature Casting of Controlled Release Microsphere," issued May 28, 1991, Patent No. 5,019,400, the teachings of which are incorporated by reference in their entirety. Methods for timed release formulation now known or later developed can be used to release the cytokine/Ig protein or plasmids into the host at a predetermine temporal point.

The vaccines described herein can be administered in various ways including intravenously (IV), intramuscularly (IM), intraperitoneal (IP), subcutaneously, and orally. Additionally, the vaccine can be administered using a transdermal patch or by inhalation therapy.

Generally, cytokines that regulate the intensity, duration and phenotype of immune responses and are involved in mediating immunity and T-cell function. Researchers have attempted to use cytokines to amplify an immune response to a vaccine. See Xiang, Z. *et al.*, *Immunity* 2: 129-135, February 1995; Tsuji, T., K. *et al.*, *J. Immunol.* 158: 4008-4013 (1997); Kim, J. J., *et al.*, *J. Immunol.* 158: 816-826 (1997). However, these studies are inconclusive because they either utilize sub-optimal or weak DNA constructs or examine only one or two of several immunologic parameters. All of these studies failed to study the effect of cytokine administration with a vaccine at systematic temporal points. Additionally, several vaccines exist which are ineffective or create an insufficient immune response.

One embodiment of the present invention encompasses a vaccine composition that has two components, a vaccine and a cytokine or a cytokine/Ig fusion protein or plasmid. The cytokine or cytokine/Ig fusion protein, or plasmid, is formulated for timed-release (e.g., encased within a timed-release drug delivery formulation) that allows the protein or plasmid to be released at a time subsequent

to vaccine administration. This time point can be predetermined to occur any time substantially immediately after vaccine administration up until about 7 days thereafter. This time point can vary depending on the type of cytokine used in the fusion protein, type of vaccine used, and the method of administration (e.g., intramuscular injection as opposed to a intravenous injection). Experiments showed that administering the IL-2/Ig protein or a GM-CSF plasmid on the second day and fifth day after vaccine administration elicited an enhanced immune response. Therefore, one embodiment of the present invention is a vaccine composition in which a timed release formulation releases the cytokine or cytokine/Ig fusion protein or plasmid on or after day 2, but before or on day 5.

Many timed release formulations are known to those of skill in the art that are suitable for delivering the cytokine/Ig fusion protein or plasmid. Some examples include microspheres, liposomes, microcapsules, implants, non-degradable materials, biodegradable material and polymers used for controlled temporal release. For example, a timed release formulation can release the cytokine/Ig fusion protein or plasmid at predetermined temporal point by varying the size of a microsphere. A smaller microsphere or similar formulation will release the cytokine/Ig fusion protein/plasmid earlier than a larger microsphere. The vehicle is only one factor to consider in constructing a timed release formulation. Other factors include the form of the fusion protein and the site of inoculation. The form of the fusion protein such as uncomplexed protein, complexed protein with a particle such as gold, or a DNA-protein conjugates, etc. all effect the absorption of the protein. Also, the tissue density of the inoculation site affects the absorption of the fusion protein/plasmid as does the blood flow to the site. These factors may affect the specific temporal point at which the fusion protein/plasmid will be released, but does not impede a skilled artisan from administering the protein/plasmid at some point subsequent vaccine administration or determining an approximate temporal point. Moreover, despite some of these factors which affect the specific time for administration of the cytokine/Ig protein or plasmid, the prior art reveals ways to accomplish administering this protein subsequent to vaccine administration without undue experimentation. See Zhao,

Z. *et al*, "Controlled Delivery of Antigens and Adjuvants in Vaccine Development," *J. of Pharmaceutical Sciences*, Vol. 85, No. 12, 1261-1270, (December 1996); Berstein *et al*, "Modulated release from Biocompatible Polymers," Patent No. 5,565,297, issue date, August 12, 1997, the teachings of both are incorporated by reference in their entirety. Additionally, the timed release formulation can release the fusion protein or plasmid in a bolus fashion or gradually. For example, liposomes and various polymer coatings can be utilized for gradual or sustained release of the cytokine/Ig fusion protein or plasmid.

The invention embodies a vaccine composition which can be used with any and all vaccines, including DNA vaccines, live organism attenuated vaccines, killed organism vaccines, subunit vaccines, recombinant or engineered protein vaccines, toxoid vaccines, peptide vaccines and live vector vaccines. The invention also encompasses vaccines known now or developed in the future.

Another aspect of the invention involves various forms of the cytokine or cytokine/Ig protein or plasmid, including those cytokines not yet identified. Cytokines suitable for use in the present invention include, for example, IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ. In one embodiment, the invention utilizes the IL-2 or the GM-CSF protein.

Plasmids which encode the cytokine/Ig fusion protein and methods of using this plasmid are specifically encompassed by the present invention. (See Example 2 for a more detailed description , Figure 8 and Figures 9A and B.) Methods for constructing a plasmid containing genes which encode a fusion protein are described in co-pending applications. Strom *et al.*, "Chimeric Cytokines and Uses thereof," Serial No. 08/431,535, filed April 28, 1995, and the parent application, Serial No. 08/355,502, filed December 12, 1994, the teachings of both are incorporated by reference in their entirety; Sytkowski, A. *et al*, "Production and use of recombinant erythropoietin/immunoglobulin fusion proteins," Serial Number 08/889,829, filed on July 10, 1997, the teachings of which are incorporated by reference in their entirety. Generally, a DNA sequence which contains the coding regions for cytokine/Ig fusion protein was inserted into a self replicating plasmid that produces the fusion protein. The resultant plasmid

contains a nucleic acid sequence that encodes a cytokine/Ig fusion protein, nucleic acid which provides an origin for plasmid replication, a promoter sequence, an operably linked termination sequence, and optionally an antibiotic resistant sequence. The cytokine/Ig fusion plasmid can also be constructed with a timed release formulation, as described herein, for release into a host after vaccine administration.

An embodiment of the claimed invention is a mammalian cytokine fusion protein. The fusion protein can comprise the following cytokines: IL-1, IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-15, TNF-α , TNF-β, IFN-γ, and GM-CSF. A preferred embodiment is an IL-2/Ig cytokine fusion protein. Accordingly, the invention includes the nucleic acid sequence that encodes the IL-2/Ig fusion protein (SEQ ID NO:1 or 3), the complement thereof, nucleic acid that hybridizes thereto under stringent conditions which can be determined using methods well known in the art, and RNA sequences transcribed from these nucleic acid sequences. The invention also pertains to vectors and host cells that contain the claimed isolated nucleic acid sequences, and probes that hybridize to these nucleic acid sequences.

An embodiment of the claimed invention includes an isolated IL-2 cytokine fusion protein comprising SEQ ID NO:2 or 4, or an amino acid sequence encoded by SEQ ID NO: 1 or 3. The claimed invention embodies an antibody specific to the IL-2/Ig fusion protein and cells that express the IL-2/Ig fusion protein.

The present invention is intended to encompass mammalian cytokine fusion proteins (e.g., IL-2/Ig), and proteins and polypeptides having amino acid sequences analogous to the amino acid sequence of mammalian cytokine fusion proteins (e.g., IL-2/Ig), and vaccine compositions having these proteins. Such polypeptides are defined herein as cytokine fusion protein analogs (e.g., homologues, IL-2/Ig protein analogs or homologues), or mutants or derivatives. Analogous amino acid sequences are defined herein to mean amino acid sequences with sufficient identity with a mammalian IL-2/Ig fusion protein's amino acid sequence to possess the biological activity of a mammalian IL-2/Ig fusion protein. For example, an analog polypeptide can be produced with "silent" changes in the amino acid sequence wherein one, or more, amino acid residues differ from the

amino acid residues of a mammalian IL-2/Ig fusion protein, yet still possesses the biological activity of the IL-2/Ig fusion protein. Examples of such differences include additions, deletions or substitutions of residues of the amino acid sequence of IL-2/Ig fusion protein. Also encompassed by the present invention are analogous polypeptides that exhibit greater, or lesser, biological activity of IL-2/Ig fusion proteins of the present invention.

The mammalian IL-2/Ig fusion protein and nucleic acid sequence include homologues, as defined herein. The homologous proteins and nucleic acid sequences can be determined using methods known to those of skill in the art. Initial homology searches can be performed at NCBI against the GenBank (release 87.0), EMBL (release 39.0), and SwissProt (release 30.0) databases using the BLAST network service. Altshul, SF, et al, Basic Local Alignment Search Tool, J. Mol. Biol. 215: 403 (1990), the teachings of which are incorporated herein by reference. Computer analysis of nucleotide sequences can be performed using the MOTIFS and the FindPatterns subroutines of the Genetics Computing Group (GCG, version 8.0) software. Protein and/or nucleotide comparisons can also be performed according to Higgins and Sharp (Higgins, D.G. and P.M. Sharp, "Description of the method used in CLUSTAL," *Gene, 73*: 237-244 (1988)). Homologous proteins and/or nucleic acid sequences to the mammalian IL-2/Ig fusion protein and/or nucleic acid sequences that encode the mammalian IL-2/Ig fusion protein are defined as those molecules with greater than 70% sequences identity and/or similarity (e.g., 75%, 80%, 85%, 90%, or 95% homology).

The "biological activity" of mammalian cytokine fusion proteins is defined herein to mean a fusion protein that possess the function or similar binding properties of the cytokine fusion protein, but has a longer half-life than the cytokine. In particular, the biological activity of a mammalian cytokine fusion protein (e.g., IL-2/Ig) is the function of the cytokine, but the cytokine fusion protein has a greater half life and a higher avidity than the cytokine (e.g., IL-2). Preferably, the half life is at least 10 times greater than the half life of the cytokine (e.g., 20, 30, 50, 70, or even 100 times greater). For example, the IL-2/Ig fusion protein or a homolog thereof can exhibit a half life of at least between 6-48 hours

and preferably, at least between 8-24 hours (the IL-2 protein generally exhibits a half-life of between 13-85 minutes).

The claimed invention includes a vaccine composition having a mammalian cytokine fusion protein (e.g., murine or human) or a homologue or analogous protein thereof, as described herein. Accordingly, the claimed invention embodies a vaccine composition having the nucleic acid sequence (e.g., SEQ ID NO: 1 or 3) that codes for a cytokine fusion protein. The vaccine composition also comprises the cytokine fusion protein comprising the amino acid sequence of SEQ ID NO: 2 or 4, or an amino acid sequence encoded by SEQ ID NO: 1 or 3, or a homolog thereof.

Methods of enhancing the immunogenicity of a vaccine:

As described herein, for the first time it has been demonstrated that administering a cytokine or cytokine/Ig fusion protein or plasmid after administration of a vaccine amplifies the immune response, thereby enhancing the efficacy of the vaccine. Accordingly, the present invention encompasses methods of enhancing vaccine immunogenicity by administering to a host a vaccine and subsequently administering to the host a cytokine or cytokine/Ig fusion protein or plasmid. The invention embodies using all cytokines in the fusion protein construct, including those not yet identified. These methods are not limited by the particular cytokine/Ig fusion protein or plasmid. In one embodiment, the invention utilizes the IL-2 or the GM-CSF protein as the cytokine which is part of the fusion protein. Example 1 illustrates the effect of these cytokines administered subsequent to vaccination.

The invention utilizes a cytokine/Ig protein which has a longer half-life compared to native, unfused, cytokine. For example, a cytokine protein has a half-life that generally last only minutes, but usually less than an hour. Utilizing a cytokine/Ig protein increases the half-life significantly allowing the fusion protein to have an effect on the immune system for hours, even days. Also see Example 1 and Figure 2.

Administering the cytokine/Ig fusion protein or plasmid subsequent to the vaccination resulted in significantly amplified levels of various immune responses, such as antibody titer, CTL levels and levels of helper T-cells. See Example 1. As described herein, a tenfold decrease in the antibody response was demonstrated when the cytokine was administered together with the vaccine as opposed to the vaccine alone. However, the cytokine/Ig fusion protein surprisingly boosted the immune responses when administered subsequent to vaccine administration. Therefore, one embodiment of the present invention includes methods for administering a cytokine/Ig fusion protein subsequent to vaccine administration, in a range from substantially immediately after vaccination up to about 7 days after vaccination. The effective time point subsequent to the vaccination may vary depending on the vaccine used, the cytokine used in the fusion protein, and the method of administration used (e.g., IM as opposed to IV). The experiments performed show that an IM injection of the IL-2/Ig plasmid given on the second day and the fifth day after or a daily IP injection of the IL-2/Ig protein HIV DNA vaccine administration illustrated significant enhancement of the immune response. See Example 1. Therefore, one embodiment encompasses cytokine/Ig fusion protein or plasmid administration on the second and/or fifth day after administration of the vaccine.

The invention embodies enhancing the immunogenicity of any and all vaccines, including DNA vaccines, live organism attenuated vaccines, killed organism vaccines, subunit vaccines, recombinant or engineered protein vaccines, toxoid vaccines, peptide vaccines and live vector vaccines. The invention also encompasses vaccines known now or developed in the future. The invention embodies these various forms of vaccines because cytokine/Ig fusion administration subsequent to vaccination impacts the processes of immune system and how the immune system reacts with the vaccine and not necessarily the vaccine itself.

Additionally, the invention embodies vaccines of various seroconversion levels and cellular immune activity. Seroconversion levels and T-cell activity indicate the vaccine potency or lack thereof. Previous reports concentrated on

primarily sub-optimal vaccines to which an immune response is more easily enhanced. The present invention unexpectedly enhances potent vaccines as well as sub-optimal vaccines. Surprisingly, a subsequent IL-2/Ig fusion protein administration enhanced the immune response by tenfold with an already potent HIV vaccine having a >90% seroconversion rate in mice as compared with vaccine administration alone. See Example 1 and Figure 3. Therefore, another embodiment of the invention encompasses enhancing the level of an already potent vaccine, as well as a sub-optimal vaccine, by administering a cytokine/Ig fusion protein or plasmid subsequent to vaccine administration.

Another aspect of the invention utilizes the vaccines which immunize against all diseases, now known or later discovered, which are directly or indirectly caused by a pathogen, organism, virus, bacteria or parasite. Several problems exist in the art in which a vaccine cannot illicit an adequate immune response. Such examples include AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, influenza and treatment of cancer. The invention resolves the problem of an inadequate immune response by the administration of a cytokine/Ig fusion protein or plasmid subsequent to vaccination because this method can significantly enhance such a response.

Yet another aspect of the invention utilizes the cytokine/Ig administration, as described in the methods herein, to augment, or enhance, or at least one parameter of an immune response. One embodiment of the invention is a method for enhancing an immunologic response by administering a vaccine and subsequently administering a cytokine/Ig fusion protein or plasmid. One, or more, of the following immune response parameters are enhanced: an antibody level, a cytotoxic T-lymphocyte level or a helper T-cell level. Experiments illustrated an increased level of each of these immunologic parameters with subsequent IL-2/Ig protein administration after vaccination with an HIV DNA vaccine. See Example 1. Therefore, one embodiment encompasses augmentation of at least one of these immunologic responses and possibly two or all three.

Also encompassed by the present invention is a method of enhancing vaccine immunogenicity comprising administering to a host a vaccine, (e.g., DNA

vaccine) and subsequently administering a cytokine/Ig fusion protein or plasmid, (e.g., IL-2) wherein "subsequently" can be further defined as a range from immediately after vaccination up to day 7, and more specifically day 2 and/or day 5 and wherein at least one immunologic parameter can be augmented, e.g., antibody level, cytotoxic T-lymphocyte level and/or a helper T-cell level.

The invention additionally encompasses a method of modulating, enhancing or suppressing an immune response of a host by administering the cytokine/Ig fusion protein or plasmid, as discussed in this document. Administering the fusion protein or plasmid can augment, suppress or otherwise modulate specific immunologic parameters. For example, administration of IL-2/Ig fusion protein or plasmids augments certain immunologic parameters, whereas IL-10/Ig fusion proteins or plasmids suppresses immunologic parameters. IL-4/Ig has the ability to modulate an immune response by shifting a T_H1 response to a T_H2 response. Additionally, this type of modulation is beneficial in treatment for various types of diseases including autoimmune diseases, infectious diseases, inflammatory diseases, neoplastic diseases, and immunologic diseases.

The invention further comprises methods for administering biological response modifiers or immuno-stimulants (e.g., IL-2 therapy). Currently, IL-2 therapy is administered to patients to stimulate their immune response. However, this therapy causes several side effects and toxicity, such as capillary leak syndrome, hypotension, cardiovascular toxicity, pulmonary edema, renal toxicity, bone marrow suppression, central nervous system toxicity and/or skin toxicity. Essentially, IL-2 therapy causes large scale release of other cytokines which causes increased vascular permeability. These toxicities are, in fact, due to the short half life of IL-2. The short half life requires that high doses of IL-2 be administered to maintain therapeutic levels.

The invention significantly reduces these side effects because the IL-2/Ig fusion protein has a longer half life. Administering a IL-2/Ig fusion protein reduces these side effects because it is administered less frequently, and at lower levels. For example, the half life of the IL-2/Ig fusion protein allows for administration on a daily basis instead of on a continuous basis (e.g., intravenous

basis) as with IL-2. Additionally, the dimer structure of IL-2/Ig fusion protein produces a higher avidity (e.g., higher binding affinity to an antigen). Therefore, the IL-2/Ig fusion protein is more effective, and simply works better than the IL-2 counterpart. Accordingly, administration of the IL-2/Ig fusion protein results in lower, less frequent doses of the protein, thereby reducing the toxic side effects associated with high levels of IL-2 therapy.

An embodiment of the invention is methods for treating patients having a disease that is treated with receiving biological response modifiers. Examples of such diseases include cancer (e.g., kidney or skin cancer), immunodeficiency diseases (e.g., AIDS) and chronic forms of infectious diseases. Accordingly, the invention encompasses administering an effective amount of the IL-2/Ig fusion protein to a patient having such a disease.

Kits for enhancing the immunogenicity of a vaccine:

The vaccine compositions and cytokine compositions described herein, to be used in the methods as described herein can be encompassed within a kit. Therefore, an embodiment of the claimed invention is a kit having a vaccine as well as a cytokine/Ig fusion protein or plasmid that is encased within a timed-release drug delivery formulation. The kit encompasses the various types of applicable cytokines, vaccines and diseases also described in this document.

As described herein, the immunologic effects of co-administering protein and plasmid cytokines with an HIV-1 gp120 DNA vaccine in mice. Administering plasmid cytokines before or with gp120 DNA decreased gp120-specific antibody titers and T cell functional activity, whereas administering plasmid cytokines after gp120 DNA augmented gp120-specific immune responses. These results demonstrate that antigen-cytokine timing is a critical parameter in determining the overall biologic effect of the cytokine. Moreover, IL-2/Ig was significantly more effective than IL-2 in augmenting DNA vaccine-elicited immune responses, indicating that the Ig fusion markedly enhances the adjuvant properties of this cytokine.

Although administration of the IL-2/Ig plasmid before or with pV1J-gp120 led to markedly diminished gp120-specific immune responses, these animals nevertheless showed high levels of nonspecific cellular proliferation (Figure 5B). These data suggest that IL-2/Ig exposure to a naive immune system leads to a high level of non-specific cellular activation, above which a specific immune response is elicited poorly. In contrast, IL-2/Ig exposure to an immune system that has recently been primed with a specific antigen leads to augmentation of the specific immune response. Il-2/Ig therefore appears to amplify the existing cellular immune repertoire. Since a similar result was obtained for GM-CSF, it is likely that immunostimulatory cytokines in general operate in this fashion. In fact, this sequence of events probably recapitulates the immunology of an acute infection: first the immune system is primed by an antigen, and then a nonspecific cytokine cascade amplifies the specific response to this antigen.

The present invention is illustrated by the following examples, which are not intended to be limited in any way.

EXAMPLE 1: PLASMID CONSTRUCTION AND EVALUATION

Plasmids were constructed using standard molecular biological techniques (Sambrook, J., *et al.*, Molecular cloning: A laboratory manual." Cold Spring Harbor Laboratory Press, Plainview, New York (1989)). Polymerase chain reactions were carried out using Pfu DNA polymerase (Stratagene, La Jolla, CA), synthetic oligonucleotide primers (Operon Technologies, Alameda, CA), and a Perkin Elmer temperature cycler. Reaction conditions included 100 ng template, 250 ng of each primer, 0.2 mM dNTPs, and 2.5 U Pfu enzyme in a 100 ml volume. Cycling was performed at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 3 minutes for 25 cycles, followed by a 10 minute final extension at 72°C. PCR products were purified by gel electrophoresis and GeneClean (Bio 101, La Jolla, CA). Restriction enzymes, T4 DNA Ligase, and Bacterial Alkaline Phosphatase were purchased from Gibco BRL (Gaithersburg, MD) and used according to the manufacturer's protocols. Competent DH5a *E. coli* were transformed and plated overnight on LB plates containing 100 mg/ml ampicillin or

50 mg/ml kanamycin (Sigma, St. Louis, MO). Single colonies were picked and grown in 2 ml liquid cultures. Plasmid clones were screened by diagnostic restriction digestion and confirmed by dideoxy sequencing using synthetic oligonucleotide primers (Operon Technologies, Alameda, CA) at the Beth Israel Deaconess Medical Center Molecular Medicine sequencing facility.

Inoculated cultures of LB broth containing appropriate antibiotics were grown overnight with shaking at 37°C. Minipreparations of plasmids were performed using the Wizard DNA Purification Systems (Promega, Madison, WI). Maxipreparations of plasmids were carried out by standard alkaline lysis followed by double CsCl gradient banding. A 1 L overnight bacterial culture was centrifuged, and the pellet was resuspended in 30 ml solution I (50 mM glucose, 25 mM Tris-Cl pH 8, 10 mM EDTA pH 8). The suspension was then lysed using 30 ml solution II (1% SDS, 0.2 M NaOH), neutralized using 30 ml solution III (5 M KOAc), and then centrifuged at 3000 rpm for 30 minutes in a Sorvall centrifuge. The supernatant was removed and filtered, and 0.6 volumes of isopropanol was added. Following a 30 minute incubation and centrifugation at 10,000 rpm for 30 minutes in a Sorvall centrifuge, the supernatants were discarded and the isopropanol pellets were air dried and resuspended in 4 ml TE buffer. 4.7 g optical grade CsCl (Gibco BRL, Gaithersburg, MD) and 0.3 ml 10 mg/ml ethidium bromide were added, and the solution was ultracentrifuged at 55,000 rpm overnight at 20°C. The CsCl-banded DNA was removed and then spun on a second CsCl gradient. Following double CsCl banding, the ethidium was extracted five times using water-saturated isobutanol, and the DNA was precipitated with 0.1 volume NaOAc and 3 volumes of ethanol. The DNA was washed with 70% ethanol, resuspended in TE, extracted with phenol/chloroform, extracted with chloroform, reprecipitated with ethanol, washed with 70% ethanol, and then resuspended in sterile 150 mM NaCl. The DNA was then used for diagnostic digestions, *in vitro* transfections, or injections into mice. The final DNA had an OD 260nm/280nm ratio of 1.90-1.95.

Expression levels of plasmid constructs were tested using transiently transfected COS cells. COS cells were split to a density of 10^6 cells/100mm plate,

grown for 24 hours, and transfected with 10 mg plasmid with the calcium phosphate method using the CellPhect kit (Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. After two days cell supernatants were removed and analyzed for the presence of secreted proteins by ELISA (Endogen, Cambridge, MA).

8-12 week old female Balb/c and C3H mice were purchased from Charles River Laboratories (Wilmington, MA) or Jackson Labs (West Grove, PA). Mice were immunized as described (Shiver, J. W., *et al.*, "Humoral and cellular immunities elicited by HIV-1 DNA vaccination," *J. Pharm. Sci.*, 85: 1317-1324 (1996)). Briefly, mice were injected intramuscularly in the quadriceps with 10-200 mg plasmid DNA encoding gp120 or cytokine genes in 100 ml 150 mM sterile saline with no adjuvant. Half the dose was given in each leg. Soluble IL-2/Ig protein was prepared as described (Zheng, X. X., *et al.*, "Administration of noncytolytic IL-10/Fc in murine models of lipopolysaccharide-induced septic shock and allogeneic islet transplantation," *J. Immunol.*, 154: 5590-5600 (1995)). Mice receiving IL-2 (BioSource, Camarillo, CA) or IL-2/Ig were given daily intraperitoneal injections of 0.3-1 mg protein in 100 ml PBS. Certain groups of mice were boosted after 2-3 months with 50 mg pV1J-gp120.

EXAMPLE 2: ANTI-GP 120 ELISA ASSAY

A direct ELISA was used to measure serum titers of murine anti-gp120 antibodies. 96-well Maxisorp ELISA plates (Nunc, Naperville, IL) were coated overnight at 4°C with 100 ml of 1 mg/ml recombinant human gp120 (Intracel, Cambridge, MA) in PBS. The remainder of the ELISA was carried out at room temperature. Following a wash with PBS containing 0.05% Tween-20, the wells were blocked for 2 hours with a solution containing 2% bovine serum albumin (Sigma, St. Louis, MO) and 0.05% Tween-20 in PBS. Sera were prepared from murine bleeds, serially diluted in 2% BSA/0.05% Tween-20, and added to ELISA wells. Following a 1 hour incubation, the plate was washed three times and then incubated with a 1:5000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary antibody (Jackson Laboratories, West Grove, PA) in 2%

BSA/0.05% Tween-20 for 1 hour. The plate was washed three times, developed with TMB (KPL, Gaithersburg, MD), stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader. Subtyping of antibodies was carried out using the Clonotyping System (Southern Biotech, Birmingham, AL) using the manufacturer's protocols.

EXAMPLE 3: PREPARATION AND STIMULATION OF MURINE SPLENOCYTES

Spleens from the DNA-vaccinated mice were aseptically removed and single cell suspensions were prepared using a No. 100 surgical stainless steel mesh. Red blood cells were removed by treating the spleen cells with NH_4Cl-KCl lysis buffer for 5 minutes at 4°C followed by two washes in Hank's Balanced Salt Solution containing 2% calf serum.

Normal Balb/c splenocytes were incubated with 40 mM gp120 IIIB P18 peptide (RIQRGPGRAFVTIGK, Multiple Peptide Systems, San Diego, CA) for 2 hours at 37°C and then irradiated in a GammaCell irradiator. $5x10^7$ splenocytes from DNA-vaccinated mice were stimulated with $5x10^6$ peptide-pulsed and irradiated normal syngeneic splenocytes in 12-well tissue culture plates (Falcon, Becton-Dickinson, Mountain View, CA) in 2 ml RPMI 1640 containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 20 U penicillin/ml, 20 mg streptomycin/ml, and $5x10^{-5}$ M 2-mercaptoethanol (all from Life Technologies, Grand Island, NY). The splenocytes were incubated at 37°C, 5% CO_2 for 6 days. Effector cells were harvested from the culture on the 7th day and used in a ^{51}Cr cytotoxicity assay.

EXAMPLE 4: ^{51}Cr RELEASE CYTOTOXICITY ASSAY

This assay was performed as described (Shiver, J. W., *et al.*, "Cytotoxic T lymphocyte and helper T cell responses following HIV polynucleotie vaccination," *Ann. NY Acad. Sci.*, 772: 198-208 (1995); and Shiver, J. W., *et al.*, "Humoral and cellular immunities elicited by HIV-1 DNA vaccination," *J. Pharm. Sci.*, 85: 1317-1324 (1996)) using the mastocytoma cell line P815 as target cells. P815 cells

were pulsed overnight with 40 mM P18 peptide at 37°C, 5% CO_2 and labeled with 150 mCi ^{51}Cr (ICN Biomedicals, Irvine, CA) for 90 minutes at 37°C, 5% CO_2. After three washes, the radiolabeled target cells were resuspended in complete RPMI 1640 at a concentration of $1x10^5$ cells/ml. The effector cells in a total volume of 100 ml were added in duplicate into the wells of a 96-well, U-bottomed tissue culture plate (Falcon, Lincoln Park, NJ). After a 5 hour incubation at 37°C, 5% CO_2, 50 ml supernatants were harvested from each well, mixed with scintillation fluid, and measured using a Wallac 1450 Microbeta liquid scintillation counter. To measure spontaneous release of ^{51}Cr, target cells were incubated with 100 ml of medium, and for maximum release target cells were incubated with 100 ml of 10% Triton X-100 in PBS. Spontaneous release in each experiment was approximately 10% of the maximum release. The percent specific cytotoxicity was calculated as: (experimental release - spontaneous release) / (maximum release - spontaneous release).

EXAMPLE 5: PROLIFERATION ASSAY

[^3H]-TdR uptake assay was used to measure the proliferation of splenocytes after antigenic stimulation. Splenocytes from DNA-vaccinated animals were resuspended at a concentration of $4x10^6$ cells/ml in RPMI 1640 containing 5% fetal bovine serum and antibiotics as described above. 100 ml of the cell suspension was added to each well of a 96-well flat-bottom tissue culture plate. Recombinant HIV-1 gp120 (Intracel, Cambridge, MA) was added at a final concentration of 2.0 mg/ml, 0.4 mg/ml, 0.1 mg/ml, or 0 mg/ml. After 4 days of culture, 1 mCi [^3H]-TdR (ICN Biomedicals, Irvine, CA) was added to each well and incubated overnight at 37°C, 5% CO_2. The cells were then harvested on glass filter paper using a Tomtec cell harvester, and the radioactivity present in the cells was measured in a Wallac 1450 Microbeta liquid scintillation counter.

EXAMPLE 6: CYTOKINE ELISA ASSAYS

$4x10^6$ splenocytes from the experimental animals were cultured with 2 mg/ml recombinant gp120 (Intracel, Cambridge, MA) in a total volume of 1 ml

RPMI 1640 containing 5% fetal bovine serum in a 24-well tissue culture plate for 72 hours. The supernatants were harvested and assayed for the presence of cytokines using ELISA kits (Endogen, Cambridge, MA) according to the manufacturer's protocol.

EXAMPLE 7: IMMUNOGENICITY OF DICISTRONIC DNA VACCINES COEXPRESSING gp120 AND A CYTOKINE

Studies were initiated in order to explore the use of plasmid-expressed cytokines as a strategy for amplifying immune responses elicited by plasmid DNA vaccines. pV1J-gp120, a DNA vaccine encoding HXBc2 gp120 IIIB, has previously been shown to elicit potent humoral and cellular immune responses in mice and nonhuman primates (Shiver, J. W., *et al.*, "Humoral and cellular immunities elicited by HIV-1 DNA vaccination," *J. Pharm. Sci.*, 85: 1317-1324 (1996); and Montgomery, D. L., *et al.*, "Heterologous and homologous protection against influenza A by DNA vaccination: Optimization of DNA vectors," *DNA and Cell Bio.*, 12: 777-783 (1993)). This vaccine is derived from pUC19 with a kanamycin resistance gene, a cytomegalovirus (CMV) IE1 enhancer, promoter, and intron A, the gene encoding gp120, and a bovine growth hormone (BGH) polyadenylation sequence (Chapman, B. S., *et al.*, "Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells," *Nuc. Acids Res.*, 19: 3979-3986 (1991)). In order to examine the effects of plasmid-expressed cytokines on immune responses to pV1J-gp120, three dicistronic vaccines were constructed from pV1J-gp120 using standard molecular biological methods (Sambrook, J., *et al.*, Molecular cloning: A laboratory manual." Cold Spring Harbor Laboratory Press, Plainview, New York (1989)). These vaccines included the pV1J backbone with both gp120 and a cytokine gene, either IL-2, IL-4, or GM-CSF. The gp120 and cytokine genes were separated in these constructs by the encephalomyocarditis virus internal ribosome entry site (IRES), which has been shown to promote efficient internal initiation of translation (Davies, M. V. and R. J. Kaufman, "The sequence context of the initiation codon in the encephalomyocarditis virus leader modulates

efficiency of internal translation initiation," *J. Virol.*, 66: 1924-1932 (1992)).

The pV1J-gp120 control, pV1J (sham), pV1J-gp120/IL-2, pV1J-gp120/IL-4, and pV1J-gp120/GM-CSF vaccines were tested for *in vitro* protein expression levels. COS cells were transiently transfected with the constructs, and cell supernatants were analyzed after 2 days by ELISA for the presence of gp120 and cytokines. As shown in Table 1, the pV1J (sham) negative control plasmid had no detectable expression of gp120, whereas the monocistronic pV1J-gp120 and the dicistronic pV1J-gp120/cytokine plasmids all had comparable high expression levels of gp120. The pV1J-gp120/cytokine constructs also expressed the appropriate cytokine, and the molar ratio of gp120 to cytokine expression for all constructs was 1.5-2.0 to 1.

Groups of Balb/c mice (N=10 per group) were then immunized with 100 mg or 10 mg of either the monocistronic pV1J-gp120 vaccine or the dicistronic pV1J-gp120/cytokine vaccines. Specifically the mice were immunized i.m. with 100 μg or 10 μg pV1J-gp120 control, pV1J-gp120/IL-2, pV1J-gp120/IL-4, or pV1J-gp120/GM-CSF. The plasmids, dissolved in sterile saline without adjuvant, were injected in both hind legs in the quadriceps muscle. Four weeks later, the mice were bled, and sera were tested by ELISA for the presence of anti-gp120 antibodies. As shown in Figure 1, a single inoculation of the control pV1J-gp120 vaccine elicited a strong anti-gp120 antibody response. The seroconversion frequency in the mice was over 90%. Surprisingly, the mice receiving the dicistronic gp120/IL-2 and gp120/IL-4 vaccines developed antibody responses more than tenfold weaker than those receiving the control gp120 vaccine, despite the similar expression levels of all the constructs *in vitro*. The mice receiving the dicistronic gp120/GM-CSF vaccine developed antibody responses that were weaker than but within the experimental error of the monocistronic gp120 vaccine.

Table 1. *In vitro expression levels of dicistronic pV1J-gp120/cytokine constructs in transiently transfected COS cells.*

V1J Construct	In vitro expression levels		gp120 : cytokine ratios	
	gp120	cytokine	mass ratio	molar ratio
blank (sham)	0 mg/ml	0 ng/ml	-	-
gp120	1.0 mg/ml	0 ng/ml	-	-
gp120 / IL-2	0.7 mg/ml	50 ng/ml	14	2.0
gp120 / IL-4	1.2 mg/ml	100 ng/ml	12	1.5
gp120 / GM-CSF	1.0 mg/ml	75 ng/ml	13	1.6

10^7 COS cells in 10 mls RPMI/5% FCS medium were transfected with 3 mg of each of the constructs using the calcium phosphate method (CellPhect, Pharmacia). After 3 days the supernatants were analyzed for secreted gp120 or cytokines by ELISA (Endogen). Results shown are means of two separate transfections each assayed in duplicate.

EXAMPLE 8: EFFECTS OF SOLUBLE IL-2 PROTEIN AND SOLUBLE IL-2/Ig FUSION PROTEIN ON THE ANTI-gp120 IMMUNE RESPONSES ELICITED BY pV1J-gp120

IL-2 has previously been characterized as a factor that augments rather than suppresses specific immune responses, and it has been shown to be an effective adjuvant for subunit and inactivated virus vaccines (Kashima, N., *et al.*,. "Unique structure of murine interleukin-2 as deduced from cloned cDNAs," *Nature (Lond.)*, 313: 402-404 (1985); Weinberg, A. and T. C. Merigan, "Recombinant interleukin-2 as an adjuvant for vaccine-induced protection. Immunization of guinea pigs with herpes simplex virus subunit vaccines," *J. Immunol.*, 140: 294-299 (1988); and Nunberg, J. H., *et al.*, "Interleukin-2 acts as an adjuvant to increase the potency of inactivated rabies virus vaccine," *Proc. Natl. Acad. Sci. USA.*, 86: 4240-4243 (1989)). Therefore, further experiments were conducted to investigate the effects of this cytokine on the immune response elicited by pV1J-gp120. It was first examined whether soluble IL-2 protein administered systemically following vaccination would modulate the anti-gp120 antibody response. Groups of Balb/c

mice (N=4 per group) were immunized with 50 mg pV1J-gp120 plus daily intraperitoneal injections of either PBS alone or 0.4 mg IL-2 in PBS for 20 days following vaccination. After 4 weeks mice were bled, and sera were tested for specific anti-gp120 antibodies by ELISA. Figure 2A demonstrates that the anti-gp120 antibody response elicited by pV1J-gp120 was not significantly altered by IL-2 administration. The soluble IL-2/Ig protein but not soluble IL-2 protein administered systemically enhances the humoral immune response to the DNA vaccine pV1J-gp120.

It is reasonable to believe that this lack of effect may be explained by the brief circulatory half-life of IL-2. The IL-2/Ig was expressed and purified. This fusion protein has a much longer half-life *in vivo* and also acts as divalent IL-2 (Zheng, X. X., *et al.*, "Administration of noncytolytic IL-10/Fc in murine models of lipopolysaccharide-induced septic shock and allogeneic islet transplantation," *J. Immunol.*, 154: 5590-5600 (1995); and Nickerson, P., *et al.*, "Prolonged islet allograft acceptance in the absence of interleukin 4 expression," *Transpl. Immunol.*, 4: 81-85 (1996)). A similar experiment was then performed to examine the effects of soluble IL-2/Ig protein on the immune response elicited by pV1J-gp120. Groups of Balb/c mice (N=8 per group) were immunized with either 50 mg pV1J-gp120 or 50 mg pV1J (sham) plasmid. Two groups of mice receiving pV1J-gp120 also received daily intraperitoneal injections of either 1 mg Ig control protein or 1 mg IL-2/Ig in PBS. (1 mg IL-2/Ig represents a molar equivalent to 0.4 mg IL-2.) After 4 weeks mice were bled, and sera were tested for specific anti-gp120 antibodies by ELISA. The soluble IL-2/Ig protein but not soluble IL-2 protein administered systemically enhances the humoral immune response to the DNA vaccine pV1J-gp120. Figure 2B demonstrates that the anti-gp120 antibody response elicited by pV1J-gp120 was not altered by injection of the Ig control protein; it was, however, enhanced over tenfold by administration of IL-2/Ig.

Groups of Balb/c mice were immunized and treated with Ig control protein or IL-2/Ig as described in Figure 2. The mice were boosted after three months with 50 mg pV1J-gp120 or 50 mg pV1J (sham) plasmid without cytokine treatment and were sacrificed 4 weeks later. $4x10^5$ splenocytes were cultured in triplicates

in 100 μl RPMI/5% FCS culture medium containing 2, 0.4, 0.08, or 0 μg/ml recombinant gp120 (Repligen). After 3 days, 1 μCi/well of ^{3}H-thymidine was added, and incorporation was measured 12 hours later by a liquid scintillation counter. Four weeks later the mice were bled, and sera were tested again for anti-gp120 antibody titers. Increased titers were observed, and the IL-2/Ig group maintained over a tenfold higher antibody titer than the control group. The mice were sacrificed and recombinant gp120-specific splenocyte proliferation was assessed by standard thymidine incorporation assays. As shown in Figure 3, the splenocytes of the mice that received IL-2/Ig had higher levels of both specific and nonspecific proliferation than that of the control mice.

Cytotoxic T lymphocyte (CTL) activity in the boosted animals was assessed using splenocytes that were cultured with peptide-pulsed irradiated syngeneic antigen presenting cells. The peptide used in these studies was the H-2^{d}-restricted immunodominant V3 loop epitope of HIV-1 gp120 IIIB (RIQRGPGRAFVTIGK) (Takahashi, H., *et al.*, "Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant," *Science (Wash. DC)*, 255: 333-336 (1992)). Figure 4 shows that the soluble IL-2/Ig protein administered systemically enhances the CTL response to the DNA vaccine pV1J-gp120. Groups of Balb/c mice were immunized, boosted, and sacrificed as in Figures 2 and 3. $5x10^{6}$ splenocytes from immunized mice were cultured with $5x10^{6}$ peptide-pulsed irradiated naive splenocytes in 2 ml RPMI/10% FCS culture medium in 12 well plates. After 24 hours, 20 U/well IL-2 (Sigma) was added. After 6 days of culture, cells were harvested and added to ^{51}Cr-labeled peptide-pulsed P815 target cells at effector to target ratios of 25:1, 12:1, 6:1, and 3:1. After 5 hours of incubation, 50 μl of supernatant was harvested and added to 200 μl scintillation fluid, and radioactivity was counted in a liquid scintillation counter. % specific lysis was calculated as (experimental release - spontaneous release) / (maximum release - spontaneous release). Figure 4 shows effector cell killing of peptide-pulsed P815 target cells and demonstrates that specific CTL activity of the mice that received IL-2/Ig was significantly greater than that of the control mice.

Table 2 shows the cytokine secretion profiles of recombinant gp120-stimulated splenocytes from the same animals. Splenocytes from pV1J (sham)-injected mice demonstrated only low levels of cytokine expression. Splenocytes from the mice that received pV1J-gp120 plus the Ig control protein exhibited high levels of IFN-g and IL-2 expression and lower levels of IL-4 and IL-10 expression, consistent with the expected T_H1 response (Lekutis, C., *et al.*, "HIV-1 env DNA vaccine administered to rhesus monkeys elicits MHC class II-restricted CD4 T helper cells that secrete IFN-g and TNF-a," *J. Immunol.*, 158: 4471-4477 (1997)). The splenocytes from the mice that received pV1J-gp120 plus IL-2/Ig showed higher expression of IFN-g, IL-4, and IL-10.

Table 2. *Cytokine secretion profiles of splenocytes of mice receiving pV1J-gp120 plus systemic treatment with soluble Ig control protein or soluble IL-2/Ig protein.*

	pV1J sham	pV1J-gp120 + Ig	pV1J-gp120 + IL-2/Ig
IFN-g	<50	5600 ± 200	23,000 ± 3200
IL-2	200 ± 20	3700 ± 900	4200 ± 640
IL-4	<10	18 ± 4	150 ± 42
IL-10	<25	120 ± 20	2500 ± 810
TNF-a	<25	<25	<25

Mice were immunized, treated with systemic Ig control protein or IL-2/Ig, and boosted as described in Figures 2 and 3. $4x10^6$ splenocytes were cultured in 1 ml RPMI/5% FCS culture medium containing 2 mg/ml recombinant gp120 (Repligen). After 3 days, supernatants were harvested and tested for specific cytokines by ELISA (Endogen). Results shown are in pg/ml and represent means and standard errors of experiments involving 4 mice per group each assayed in triplicate.

EXAMPLE 9: EFFECTS OF PLASMID IL-2 AND PLASMID IL-2/Ig IB ABTU-gp120 IMMUNE RESPONSES ELICITED BY pV1J-gp120

In order to investigate further the effects of IL-2/Ig on DNA vaccine-

elicited immune responses, monocistronic plasmids containing either IL-2 or IL-2/Ig in the pV1J backbone were constructed using standard molecular biological methods (Sambrook, J., *et al.*, Molecular cloning: A laboratory manual." Cold Spring Harbor Laboratory Press, Plainview, New York (1989)). Expression of IL-2 and IL-2/Ig was confirmed and quantified by transient transfection experiments in COS cells followed by ELISA analysis and functional CTLL stimulation analyses using cell supernatants (data not shown). Experiments were then performed (i) to examine whether plasmid-encoded IL-2/Ig has a stimulatory effect on the vaccine-elicited immune response similar to that of soluble IL-2/Ig protein; and (ii) to clarify our findings that IL-2 administered as a dicistronic plasmid with gp120 suppressed the vaccine-induced antibody responses (Figure 1) whereas IL-2/Ig protein administered after vaccination augmented the immune responses (Figures 2-4).

Groups of Balb/c mice (N=6 per group) were immunized with 50 mg pV1J-gp120 on Day 0. Four groups of mice were also inoculated with 200 mg pV1J-IL-2/Ig but on Day -5, Day 0, Day +2, or Day +5 relative to pV1J-gp120 administration. Figure 5A demonstrates that administration of pV1J-IL-2/Ig before or with pV1J-gp120 significantly decreased the anti-gp120 antibody response. More than tenfold reduction in specific antibody titers was observed when the cytokine plasmid was administered with the gp120 plasmid on Day 0, a result similar to the reduction in the antibody response obtained with the pV1J-gp120/IL-2 dicistronic construct (Figure 1). In contrast, administration of pV1J-IL-2/Ig after vaccination with pV1J-gp120 amplified the vaccine-elicited anti-gp120 antibody response. Approximately fivefold augmentation of specific antibody titers was observed when the cytokine plasmid was administered on Day +2 relative to the gp120 plasmid.

Administering plasmid IL-2/Ig at these different times in relation to the vaccine antigen also affected the gp120-specific cellular immune responses. The mice were boosted after two months with 50 mg pV1J-gp120 without cytokine treatment and sacrificed 4 weeks later. As shown in Figure 5B, the absolute splenocyte proliferation levels were similar in all cases, but administration of

pV1J-IL-2/Ig on Day -5 or Day 0 led to significantly higher levels of nonspecific proliferation (solid bars). Figure 5C shows that pV1J-IL-2/Ig injection prior to antigen administration decreased the vaccine-elicited CTL activity, whereas pV1J-IL-2/Ig injection following the antigen increased CTL activity. Cytokine secretion profiles of cultured splenocytes, depicted in Table 3, show that all the groups of mice receiving pV1J-IL-2/Ig had slightly increased expression levels of IFN-g, IL-4, and IL-10.

In order to examine whether pV1J-IL-2, a plasmid expressing native IL-2, could also augment the immune response to pV1J-gp120, a similar experiment was performed. Groups of Balb/c mice (N=6 per group) were immunized with 50 mg pV1J-gp120 on Day 0; groups were also inoculated with 200 mg pV1J (sham) or 200 mg pV1J-IL-2 on Day +2. After 4 weeks mice were bled, and sera were tested for specific anti-gp120 antibodies by ELISA. Geometric mean titers with standard errors of total serum anti-gp120 antibodies are shown. As shown in Figure 6A, neither pV1J (sham) nor pV1J-IL-2 administered on Day +2 augmented the vaccine-elicited antibody response. In addition pV1J (sham) did not affect the antibody response when administered with pV1J-gp120 on Day 0 (data not shown). In Figure 6B, after 2 months, mice were boosted with 50 μg pV1J-gp120 and were sacrificed 4 weeks later. CTL chromium release assays were performed as described previously at effector to target ratios of 80:1, 40:1, and 20:1. Figure 6B shows that administration of pV1J-IL-2 on Day +2 also had no detectable effect on the vaccine-elicited CTL response. These results suggest that the increased immune responses observed with the IL-2/Ig plasmid (Figure 5) are specific and require the use of the IL-2/Ig fusion construct.

Table 3. *Cytokine secretion profiles of splenocytes of mice receiving pV1J-gp120 plus plasmid IL-2/Ig at different time points.*

	pV1J-gp120 alone	+ pV1J-IL-2/Ig			
		Day -5	Day 0	Day +2	Day +5
IFN-g	8500 ± 1100	11,600 ± 3300	12,600 ± 4300	14,400 ± 3100	17,100 ± 1100
IL-2	4800 ± 100	4900 ± 1800	4700 ± 900	5200 ± 1100	6400 ± 1000
IL-4	37 ± 3	110 ± 30	65 ± 21	62 ± 21	88 ± 8
IL-10	230 ± 120	1330 ± 540	990 ± 390	600 ± 240	750 ± 260
TNF-a	<25	<25	<25	<25	<25

Mice were immunized with pV1J-gp120 on Day 0 plus pV1J-IL-2/Ig on Day -5, 0, +2, or +5 as described in Figure 5. $4x10^6$ splenocytes were cultured in 1 ml RPMI/5% FCS culture medium containing 2 mg/ml recombinant gp120 (Repligen). After 3 days, supernatants were harvested and tested for specific cytokines by ELISA (Endogen). Results shown are in pg/ml and represent means and standard errors of experiments involving 6 mice per group each assayed in duplicate.

EXAMPLE 10: FURTHER EFFECTS OF ADMINISTRATION OF CYTOKINE PLASMIDS ON ANTI-gp120 IMMUNE RESPONSES ELICITED BY pV1J-gp120

Further experiments were then conducted to investigate (i) whether the timing of the administration of other plasmid cytokines relative to pV1J-gp120 is also important; and (ii) whether this phenomenon is also observed in other strains of mice. An analogous experiment to the one shown in Figure 5A was conducted using pV1J-GM-CSF as the plasmid cytokine. As shown in Figure 7A, the effects of administering pV1J-GM-CSF on the pV1J-gp120-elicited antibody response were less dramatic than those of pV1J-IL-2/Ig, but the overall trend was similar and significant. Administering pV1J-GM-CSF prior to the pV1J-gp120 antigen suppressed the vaccine-elicited antibody response, whereas administering pV1J-GM-CSF after the plasmid antigen had perhaps a mild augmenting effect. The suppressive effects observed with GM-CSF in Figure 7A are more marked than the

results observed with GM-CSF in Figure 1, possibly due to the fourfold higher dosage of cytokine administered in the experiment shown in Figure 7A.

A second experiment, also similar in design to that shown in Figure 5A was conducted with pV1J-IL-2/Ig in C3H mice rather than in Balb/c mice. As shown in Figure 7B, the effect of administering plasmid IL-2/Ig was essentially the same in both strains of mice, indicating that the previous observations were not strain-specific.

The present study differs from these previous reports in several ways. First, we have used a potent gp120 DNA vaccine that induces seroconversion in >90% of inoculated mice in the absence of cytokine augmentation (Shiver, J. W., *et al.*, "Humoral and cellular immunities elicited by HIV-1 DNA vaccination," *J. Pharm. Sci.*, 85: 1317-1324 (1996); and Montgomery, D. L., *et al.*, "Heterologous and homologous protection against influenza A by DNA vaccination: Optimization of DNA vectors," *DNA and Cell Bio.*, 12: 777-783 (1993)). Most of the previous studies have used weaker DNA vaccine constructs or have utilized cytokines to augment suboptimal immune responses. It is possible that the differences among reports partially reflects the different potencies of the baseline DNA vaccines and different levels of responsiveness to cytokines. Second, we have shown simultaneous cytokine-mediated modulation of multiple immune parameters including antibody, proliferative, CTL, and cytokine secretion activity. Third, we have compared the adjuvant properties of IL-2/Ig and IL-2, both as proteins and as plasmids, and have found significantly more augmentation with the IL-2/Ig fusion construct. Fourth, we have systematically studied the effects of changing the temporal relationship between delivery of antigen and cytokine. Simultaneous administration of plasmids expressing native cytokines with DNA vaccines, as reported previously, may be capable of enhancing the vaccine-elicited immune responses in certain instances. However, the present study suggests that this approach does not optimally harness the use of plasmid cytokines for augmenting immune responses.

EXAMPLE 11: CONSTRUCTION OF pV1J-CYTOKINE AND pVIJ-CYTOKINE/1g

Cytokine or cytokine/Ig genes were amplified by PCR using synthetic oligonucleotide primers. Although the IL-2/Ig was made using this technique, all cytokine/Ig fusion plasmids are also made the same or similar way. See Figure 9 for the DNA sequence for the coding region of the IL-2/Ig protein. The following is a method of making a plasmid which expresses the IL-2/Ig protein. After amplification, the PCR products were purified and digested with the restriction endonuclease BclI in order to generate sticky ends. The pV1J vector which was obtained from Merck, Westpoint, Pennsylvania is illustrated in Figure 8. The pV1J vector was digested with the compatible restriction endonuclease Bg1II and phosphatased with Bacterial Alkaline Phosphatase. The insert was ligated to the vector using T4 DNA Ligase, and competent DHSx E.coli were transformed with the constructs. Kanamycih-resistant colonies were picked from overnight growths on agar plates, and were grown in 2ml cultures. Following minipreparations of the plasmids from the small-scale growths, the plasmids were screened using PstI and HindIII diagnostic restriction digestions. Final plasmids were confirmed by dideoxy DNA sequencing.

EXAMPLE 12: CONSTRUCTION OF THE HUMAN IL-2/Ig PLASMID AND FUSION PROTEIN

Human IL-2 was amplified by PCR using oligonucleotide primers with the engineered restriction sites Bg1II at the 5' end and PvuI at the 3' end. The Fc portion of human IgG2a was amplified by PCR using oligonucleotide primers with the engineered restriction sites PvuI at the 5' end and Bg1II at the 3' end. The inserts were then digested with Bg1II and PvuI. The vectors pV1J and pCMV were digested with Bg1II. The pV1J-IL-2/Ig and pCMV-IL-2/Ig vectors were made by a triple ligation using the vector backbones and both the Fc and the IL-2 inserts. DNA sequences and protein expression were then confirmed. Cells expressing IL-2/Ig protein were made by transfection of NS-1 cells with the pCMV-IL-2/Ig vector, selection with the antibiotic G418 sulfate, and screening of

clones for protein expression by ELISA. Purification of human IL-2/Ig protein was performed by passing culture supernatant over protein A columns by standard methods. The nucleic acid and amino acid sequence of the human IL-2/Ig protein appears in Figure 10A-E.

EXAMPLE 13: AUGMENTATION OF IMMUNE RESPONSE TO SHIV-SPECIFIC DNA VACCINES BY IL-2/IG ADMINISTRATION OF RHESUS MONKEYS

The objective of this study is to determine the effects of administering IL-2/Ig, a fusion protein with IL-2 activity and a long *in vivo* half-life, on immune responses elicited by SHIV-specific DNA vaccines in monkeys. It has been previously shown in mice that both plasmid Il-2/Ig and soluble IL-2/Ig protein greatly augment immune responses elicited by an HIV gp120-specific DNA vaccine, as determined by antibody titers, functional CTL activity, cellular proliferative responses, and cytokine secretion levels. The current study will examine if such augmentation is also achieved in monkeys, and whether the subsequent immune responses will be sufficient to protect against a pathogenic viral challenge.

It is expected that the effect of human IL-2/Ig in monkeys will be similar to that of murine IL-2/Ig in mice, as shown in Examples 1-10. Human IL-2 and monkey IL-2 cross-react. Thus, the expectation is that human IL-2/Ig administered after DNA vaccination in monkeys will enhance vaccine-elicited immune responses, as did in the mice.

The vaccination experiment will require 12 healthy monkeys, which will be divided into 3 groups of 4 monkeys. All monkeys will be immunized i.m. with 5 mg IIV-1 89.6P gp120 DNA + 5 mg SIV gag DNA. The first group of monkeys will receive no cytokine. The second group will receive 5 mg plasmid IL-2/Ig two days following vaccination. The third group will receive multiple injections of IL-2/Ig protein for 12 weeks following vaccination. IL-2/Ig protein will be purified from approximately 100 liters of cell culture using protein A columns. Boosts will take place at 4 weeks, 8 weeks, and 24 weeks. The monkeys will be bled 3 weeks

after each vaccination and possibly more frequently. We will measure antibody titers by ELISA, neutralizing functional CTL activity. All Mamu-A*01 positive animals will also be analyzed by tetramer staining technology for generation of specific CTLs. The monkeys are challenged with SHIV-89.6P (KB9) after the 24 week boost and include several naive control monkeys. Post-challenge we will continue to study these immunological paramenters as well as viral loads, lymph node biopsies, and clinical outcomes

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. A vaccine composition comprising:
   a) a vaccine, and
   b) a timed-release formulation of a cytokine or cytokine/Ig fusion protein or plasmid wherein the formulation or device releases the cytokine protein or plasmid at one or more temporal points subsequent to vaccine administration.

2. The vaccine composition of Claim 1, wherein the formulation or device releases the cytokine protein or plasmid during at least one predetermined temporal point subsequent to vaccine administration.

3. The vaccine composition of Claim 2, wherein the formulation or device is capable of a sustained or gradual release of the cytokine protein or plasmid after the predetermine temporal point.

4. The vaccine composition of Claim 3, wherein the predetermined temporal point lies between a point substantially immediately after vaccine administration, and before day 7.

5. The vaccine composition of Claim 4, wherein the predetermined temporal point is on or after Day 2 but before or on Day 5.

6. The vaccine composition of Claim 1, wherein the formulation or device comprises a formulation or device selected from the group consisting of microspheres, liposomes, microcapsules, implants, non-degradable materials, biodegradable material and polymers used for controlled temporal release.

7. The vaccine composition of Claim 1, wherein the fusion protein comprises a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ.

8. The vaccine composition of Claim 1, wherein the vaccine is selected from the group consisting of a DNA vaccine, a live organism attenuated vaccine, killed organism vaccine, a subunit vaccine, a recombinant or engineered protein vaccine, a toxoid vaccine, a peptide vaccine and a live vector vaccine.

9. The vaccine composition of Claim 1, wherein the cytokine fusion protein is IL-2/Ig and the vaccine is a gp120 AIDS vaccine.

10. The vaccine composition of Claim 1, wherein the cytokine fusion protein is GM-CSF/Ig and the vaccine is a gp120 AIDS vaccine.

11. The vaccine composition of Claim 8, wherein the vaccine is a potent vaccine.

12. The vaccine composition of Claim 8, wherein the vaccine is a suboptimal vaccine.

13. The vaccine composition of Claim 8, wherein the DNA vaccine is a vaccine comprising at least one HIV gene.

14. The vaccine composition of Claim 13, wherein the HIV gene is gp120.

15. The vaccine composition of Claim 1, wherein the vaccine is used to elicit an immune response against a disease selected from the group consisting of AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, cancer, and influenza.

16. The vaccine composition of Claim 1, wherein the cytokine/Ig fusion protein is a human cytokine/Ig fusion protein that comprises SEQ ID NO: 2 or the amino acid sequence encoded by SEQ ID NO: 1.

17. The vaccine composition of Claim 1, further comprising a nucleic acid sequence comprising:

a) SEQ ID NO:1,

b) a DNA encoding the amino acid sequence of SEQ ID NO:2,

c) a DNA which hybridizes to a DNA according to a) or b) under stringent hybridization conditions,

d) a complement of a), or b), or

e) RNA sequences transcribed from the nucleotides of a), b), c) or d).

18. The vaccine composition of Claim 1, wherein the cytokine/Ig fusion protein is a murine cytokine/Ig fusion protein that comprises SEQ ID NO: 4 or the amino acid sequence encoded by SEQ ID NO: 3.

19. The vaccine composition of Claim 1, further comprising a nucleic acid sequence comprising:

a) SEQ ID NO:3,

b) a DNA encoding the amino acid sequence of SEQ ID NO:4,

c) a DNA which hybridizes to a DNA according to a) or b) under stringent hybridization conditions,

d) a complement of a), or b), or

e) RNA sequences transcribed from the nucleotides of a), b), c) or d).

20. Use of a vaccine composition for use in therapy, e.g., AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, cancer and influenza comprising the vaccine composition of Claim 1.

21. Use of a vaccine composition comprising a vaccine, and a timed-release formulation of a cytokine or cytokine/Ig fusion protein or plasmid, wherein the formulation or device releases the cytokine protein or plasmid at one or more temporal points subsequent to vaccine administration, for the manufacture of a medicament for treating AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, cancer and influenza in an individual.

22. Use of a vaccine composition comprising a vaccine, and a timed-release formulation of a cytokine or cytokine/Ig fusion protein or plasmid, wherein the formulation or device releases the cytokine protein or plasmid at one or more temporal points subsequent to vaccine administration, for the manufacture of a medicament for treating an autoimmune disease, an infectious disease, an inflammatory disease, a neoplastic disease, and an immunologic disease in an individual.

23. A method of vaccinating a host comprising administering the vaccine composition in Claim 1.

24. A plasmid for use as a DNA vector *in vivo* expression comprising a nucleic acid sequence that encodes a cytokine or cytokine/Ig fusion protein, operably linked to a nucleic acid sequence which provides an origin for plasmid replication, a promoter sequence, and termination sequence.

25. The plasmid of Claim 24, wherein the fusion protein comprises a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ.

26. A method for enhancing vaccine immunogenicity of a host comprising administering the plasmid of Claim 23.

27. The method of Claim 26, further comprising administering the plasmid of Claim 25 subsequent to administering a vaccine to the host.

28. The method of Claim 27, wherein the vaccine is administered to the host on day 0 and the plasmid is administered to the host on, or after day 2, but before or on day 5.

29. A method of enhancing vaccine immunogenicity comprising administering to a host a vaccine and subsequently administering to the host a cytokine or cytokine/Ig fusion protein or plasmid

30. The method of Claim 29, wherein the fusion protein comprises a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ.

31. The method of Claim 29, wherein the vaccine is selected from the group consisting of a DNA vaccine, a live organism attenuated vaccine, killed organism vaccine, a subunit vaccine, a recombinant or engineered protein vaccine, a toxoid vaccine, a peptide vaccine and a live vector vaccine.

32. The method of Claim 31, wherein the DNA vaccine is a vaccine comprising at least one HIV gene.

33. The method of Claim 30, wherein the vaccine is used to elicit an immune response against a disease selected from the group consisting of AIDS, malaria, tuberculosis, Hepatitis C, Hepatitis B, cancer and influenza.

34. The method of Claim 29, wherein the vaccine is administered to the host on day 0 and the cytokine/Ig fusion protein or plasmid is administered to the host on, or after day 2, but before or on day 5.

35. A method for enhancing an immune response to an HIV DNA vaccine comprising administering a DNA vaccine and subsequently administering a cytokine or cytokine/Ig fusion protein or plasmid, wherein one or more immunologic parameters are enhanced.

36. The method of Claim 35, wherein the immunologic parameters are selected from a group of parameters consisting of an antibody level, a cytotoxic T-lymphocyte level and a helper T-cell level.

37. The method of Claim 35, wherein the fusion protein comprises all or a portion of an Ig molecule and a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ.

38. A method of vaccinating a host comprising administering a vaccine and subsequently administering a plasmid that comprises a nucleic acid sequence which encodes a cytokine or cytokine fusion protein, operably linked to a nucleic acid sequence which provides an origin for plasmid replication, a promoter sequence, and a termination sequence.

39. The method of Claim 37, wherein the cytokine protein comprises a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α, and IFN-γ.

40. The method of Claim 38, wherein the vaccine is a DNA vaccine.

41. A method for enhancing vaccine immunogenicity comprising:

a) administering a vaccine to a host, and

b) subsequently administering to the host a cytokine or cytokine/Ig fusion protein or plasmid wherein at least one immunologic

parameter selected from the group consisting of an antibody level, a cytotoxic T-lymphocyte level and a helper T-cell level is enhanced.

42. The method of Claim 41, wherein at least two immunologic parameters are enhanced.

43. The method of Claim 41, wherein all three immunologic parameters are enhanced.

44. A method of modulating, enhancing or suppressing an immune response of a host comprising administering a plasmid which encodes cytokine or a cytokine/Ig fusion protein, wherein at least one immunologic parameter is affected.

45. The method of Claim 44, wherein an immunologic parameter is enhanced and wherein the cytokine/Ig fusion protein further comprises an IL-2 cytokine.

46. The method of Claim 44, wherein an immunologic parameter is suppressed and wherein the cytokine protein further comprises an IL-10 cytokine.

47. The method of Claim 44, wherein the immune response shifts from a T_H1 response to a T_H2 response, and wherein the cytokine protein further comprises a IL-4 cytokine.

48. The method of Claim 44, wherein the host is affected by a disease selected from a group consisting of an autoimmune disease, an infectious disease, an inflammatory disease, a neoplastic disease, and an immunologic disease.

49. A kit comprising:

a) a vaccine, and

b) a timed-release formulation of a cytokine or a cytokine/Ig fusion protein or plasmid wherein the formulation or device releases the cytokine protein or plasmid at one or more temporal points subsequent to vaccine administration.

50. The kit of Claim 49, wherein the formulation or device comprises a component selected from a group consisting of microspheres, liposomes, microcapsules, implants, non-degradable materials, and biodegradable material.

51. The kit of Claim 49, wherein the cytokine protein comprises a cytokine selected from the group consisting of IL-2, GM-CSF, IL-4, IL-6, IL-7, IL-13, IL-10, IL-12, IL-15, TNF-α and IFN-γ.

52. The kit of Claim 49, wherein the vaccine is selected from the group consisting of a DNA vaccine, a live organism attenuated vaccine, killed organism vaccine, a subunit vaccine, a recombinant or engineered protein vaccine, , a toxoid vaccine, a peptide vaccine and a live vector vaccine.

53. The kit of Claim 52, wherein the DNA vaccine is a vaccine comprising at least one HIV gene.

54. The kit of Claim 49, wherein the cytokine protein is IL-2/Ig and wherein the vaccine is a gp120 AIDS vaccine.

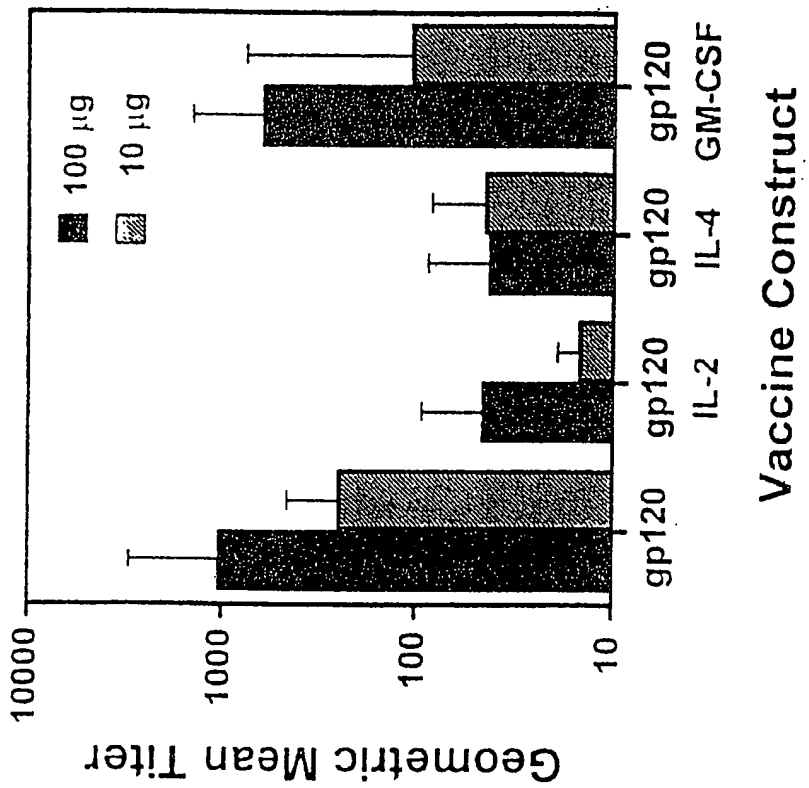
Geometric Mean Titer
10000
1000
100
10
100 µg
10 µg
gp120
gp120
gp120
gp120
IL-2
IL-4
GM-CSF
Vaccine Construct

Figure 1

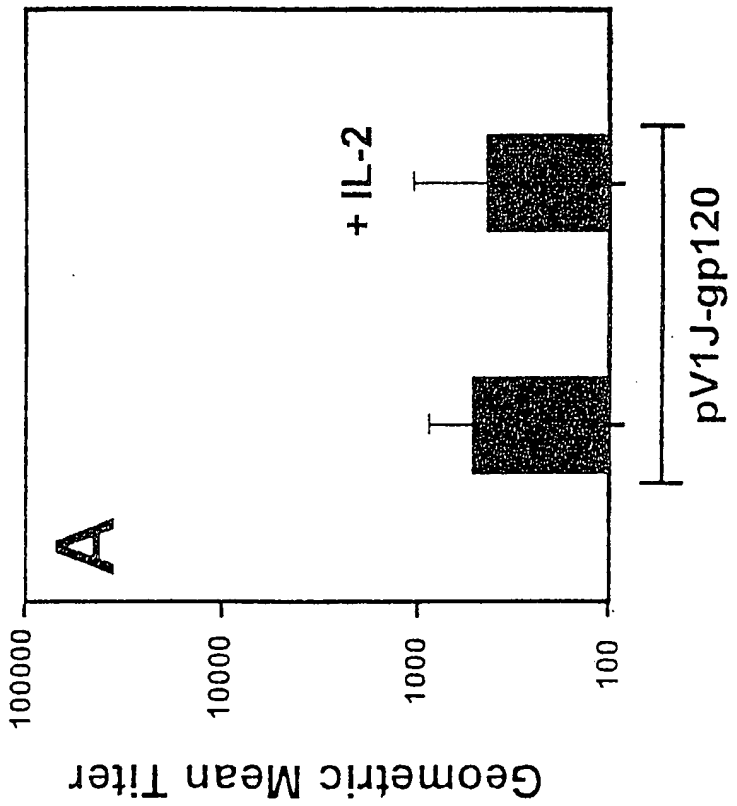

Figure 2A

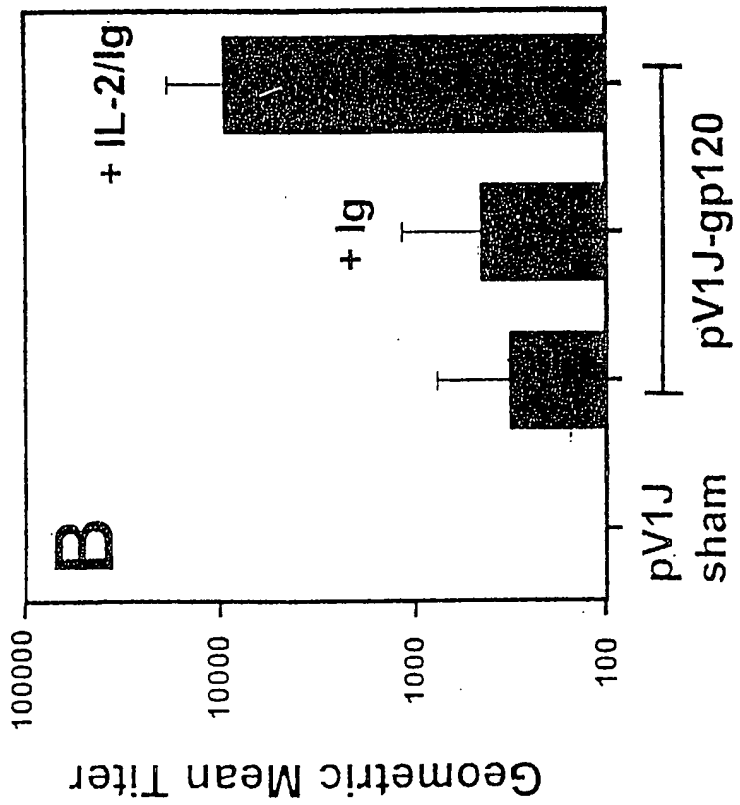
B
+ IL-2/Ig
+ Ig
pV1J
sham
pV1J-gp120
100000
10000
1000
100
Geometric Mean Titer

Figure 2B

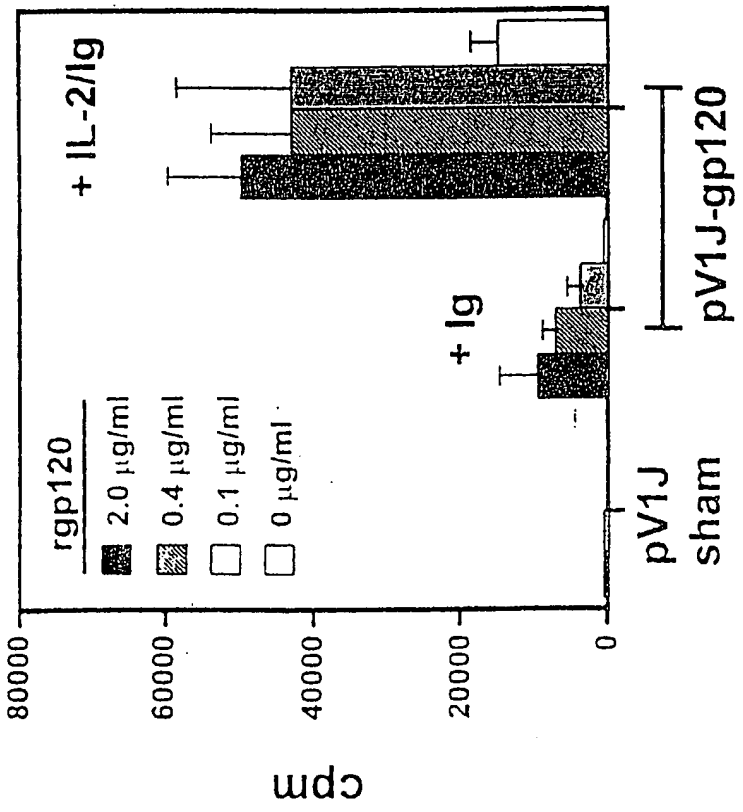
cpm
80000
60000
40000
20000
0
rgp120
2.0 µg/ml
0.4 µg/ml
0.1 µg/ml
0 µg/ml
+ IL-2/Ig
+ Ig
pV1J
sham
pV1J-gp120

Figure 3

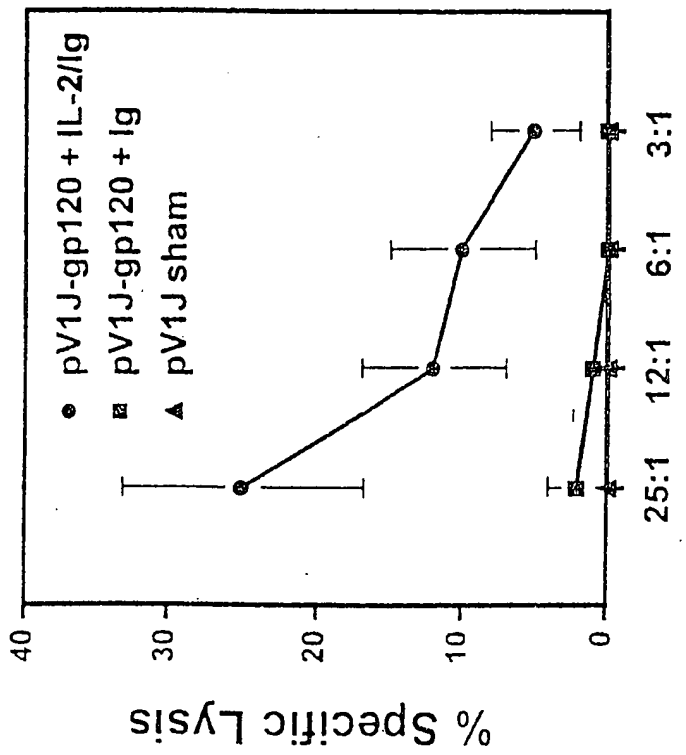
pV1J-gp120 + IL-2/Ig
pV1J-gp120 + Ig
pV1J sham
% Specific Lysis
40
30
20
10
0
25:1
12:1
6:1
3:1
E:T Ratio

Figure 4

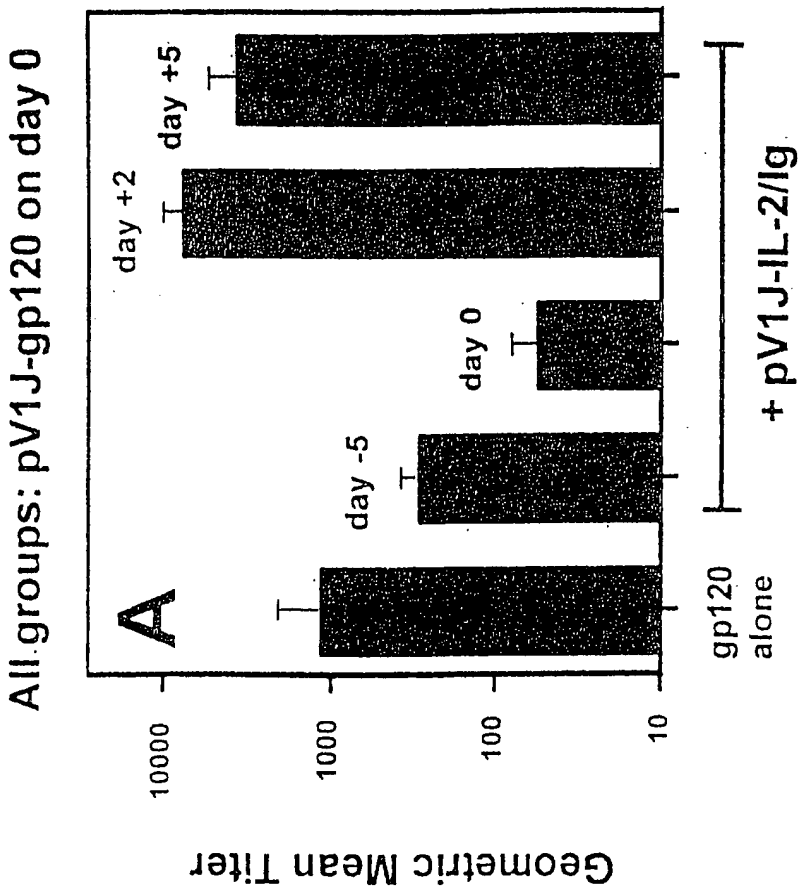
All groups: pV1J-gp120 on day 0
A
day +2
day +5
day -5
day 0
Geometric Mean Titer
10000
1000
100
10
gp120 alone
+ pV1J-IL-2/Ig

Figure 5A

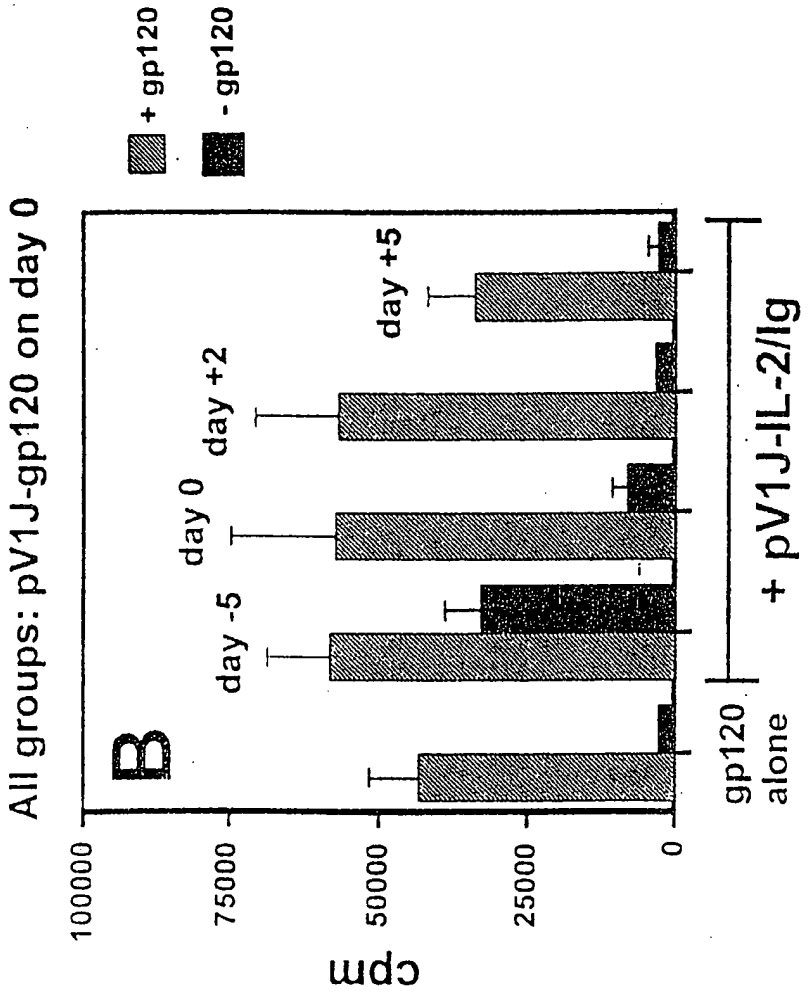
All groups: pV1J-gp120 on day 0
B
+ gp120
- gp120
day -5
day 0
day +2
day +5
cpm
100000
75000
50000
25000
0
gp120 alone
+ pV1J-IL-2/Ig

Figure 5B

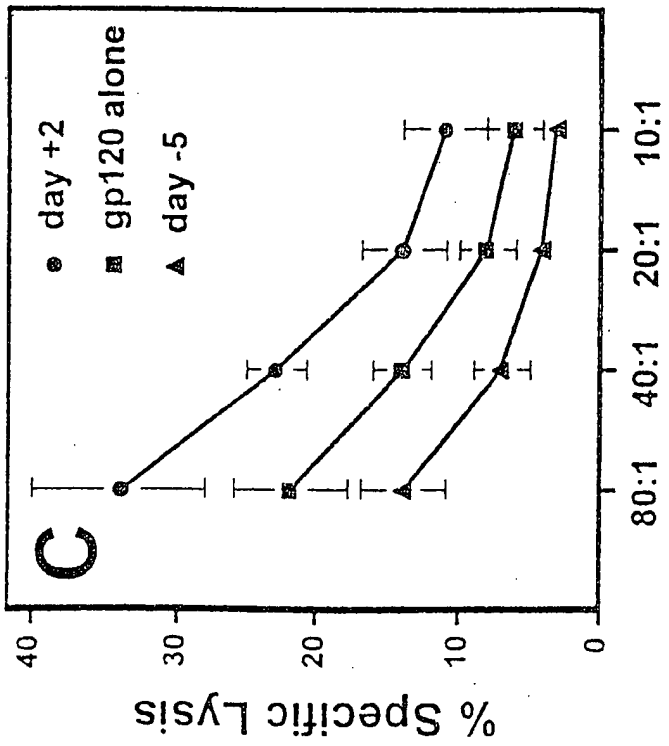
C
day +2
gp120 alone
day -5
% Specific Lysis
0
10
20
30
40
80:1
40:1
20:1
10:1
E:T Ratio

Figure 5C

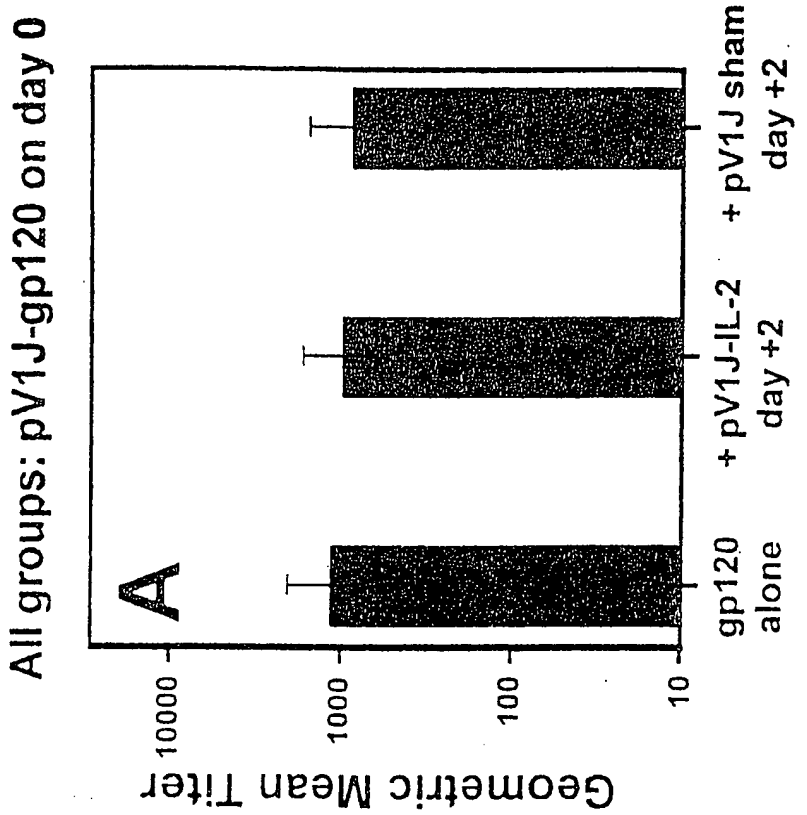
All groups: pV1J-gp120 on day 0
A
Geometric Mean Titer
10000
1000
100
10
gp120 alone
+ pV1J-IL-2 day +2
+ pV1J sham day +2

Figure 6A

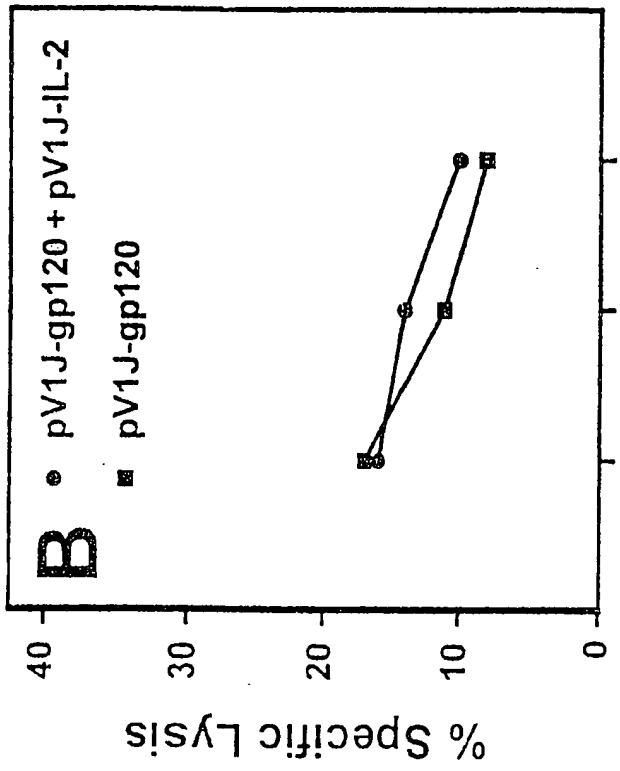
B
pV1J-gp120 + pV1J-IL-2
pV1J-gp120
% Specific Lysis
0
10
20
30
40
80:1
40:1
20:1
E:T Ratio

Figure 6B

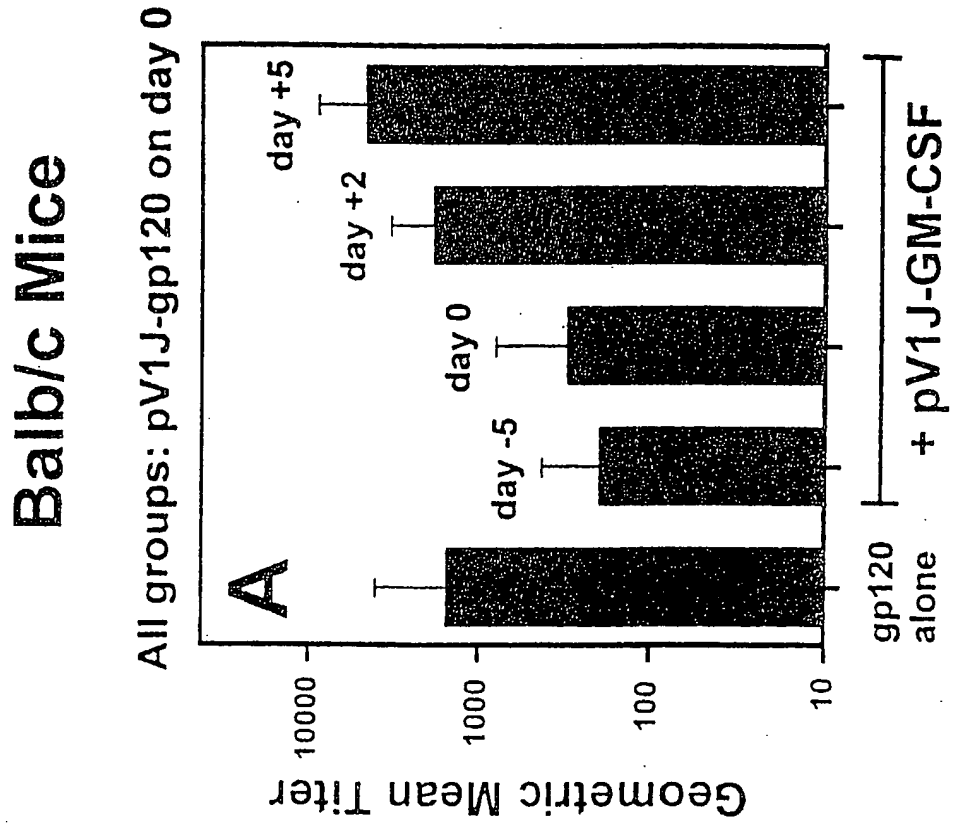
Balb/c Mice
All groups: pV1J-gp120 on day 0
A
Geometric Mean Titer
10000
1000
100
10
day -5
day 0
day +2
day +5
gp120 alone
+ pV1J-GM-CSF

Figure 7A

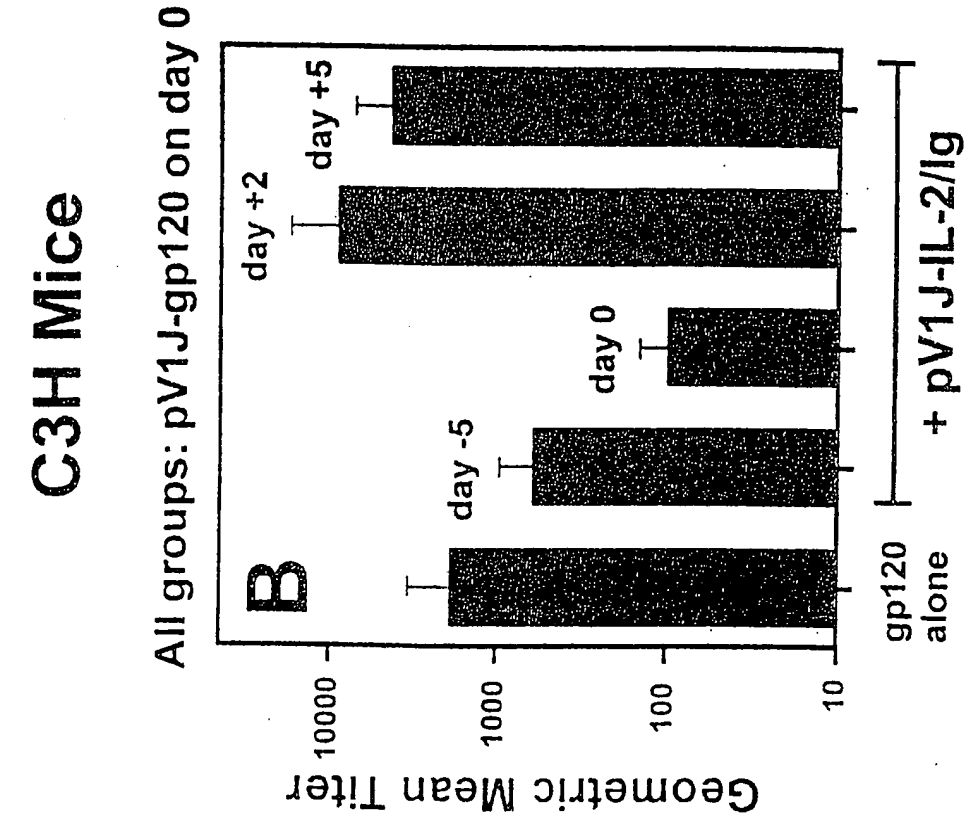
C3H Mice
All groups: pV1J-gp120 on day 0
B
day +2
day +5
day -5
day 0
Geometric Mean Titer
10000
1000
100
10
gp120 alone
+ pV1J-IL-2/Ig

Figure 7B

13/20

v1jneo
single cutters

3 May 1993
copied from gcg map

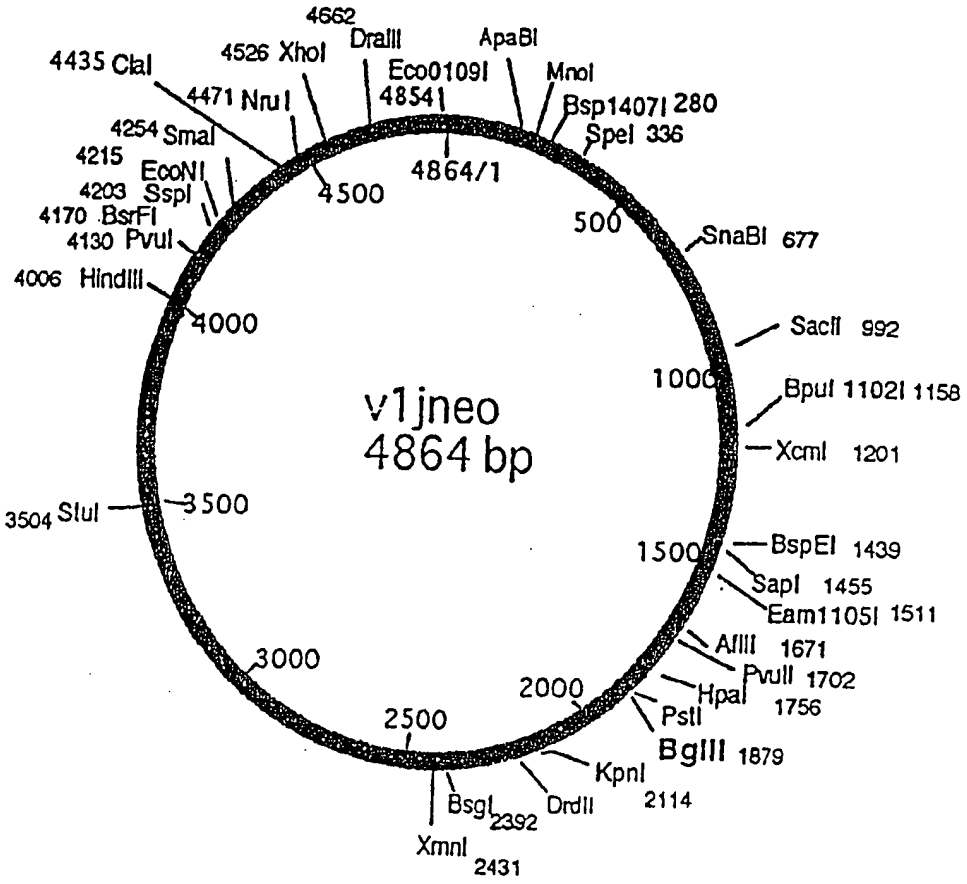

Figure 8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Leu Val Asn Ser Ala Pro Thr Ser Ser Ser Thr Ser Ser Ser Thr Ala
            20                  25                  30

GAA GCA CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAC CTG         144
Glu Ala Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln His Leu
        35                  40                  45

GAG CAG CTG TTG ATG GAC CTA CAG GAG CTC CTG AGC AGG ATC GAC AAT         192
Glu Gln Leu Leu Met Asp Leu Gln Glu Leu Leu Ser Arg Ile Asp Asn
    50                  55                  60

TAC AGG AAC CTG AAA CTC CCC AGG ATG CTC ACC TTC AAA TTT TAC TTG         240
Tyr Arg Asn Leu Lys Leu Pro Arg Met Leu Thr Phe Lys Phe Tyr Leu
65                  70                  75                  80

CCC AAG CAG GCC ACA GAA TTG AAA GAT CTT CAG TGC CTA GAA GAT GAA         288
Pro Lys Gln Ala Thr Glu Leu Lys Asp Leu Gln Cys Leu Glu Asp Glu
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CTT GGA CCT CTG CGG CAT GTT CTG GAT TTG ACT CAA AGC AAA AGC TTT         336
Leu Gly Pro Leu Arg His Val Leu Asp Leu Thr Gln Ser Lys Ser Phe
            100                 105                 110

CAA TTG GAA GAT GCT GAG AAT TTC ATC AGC AAT ATC AGA GTA ACT GTT         384
Gln Leu Glu Asp Ala Glu Asn Phe Ile Ser Asn Ile Arg Val Thr Val
        115                 120                 125

GTA AAA CTA AAG GGC TCT GAC AAC ACA TTT GAG TGC CAA TTC GAT GAT         432
Val Lys Leu Lys Gly Ser Asp Asn Thr Phe Glu Cys Gln Phe Asp Asp
    130                 135                 140

GAG TCA GCA ACT GTG GTG GAC TTT CTG AGG AGA TGG ATA GCC TTC TST         480
Glu Ser Ala Thr Val Val Asp Phe Leu Arg Arg Trp Ile Ala Phe Cys/Ser
145                 150                 155                 160

CAA AGC ATC ATC TCA ACA AGC CCT CAG CAT CCC AGA GGG CCC ACA ATC         528
Gln Ser Ile Ile Ser Thr Ser Pro Gln His Pro Arg Gly Pro Thr Ile
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```

Figure 9A

```
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Lys Pro Cys Pro Pro Cys Lys Cys Pro Ala Pro Asn Leu Glu Gly Gly
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CCA TCC GTC TTC ATC TTC CCT CCA AAG ATC AAG GAT GTA CTC ATG ATC      624
Pro Ser Val Phe Ile Phe Pro Pro Lys Ile Lys Asp Val Leu Met Ile
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TCC CTG AGC CCC ATA GTC ACA TGT GTG GTG GTG GAT GTG AGC GAG GAT      672
Ser Leu Ser Pro Ile Val Thr Cys Val Val Val Asp Val Ser Glu Asp
    210                 215                 220

GAC CCA GAT GTC CAG ATC AGC TGG TTT GTG AAC AAC GTG GAA GTA CAC      720
Asp Pro Asp Val Gln Ile Ser Trp Phe Val Asn Asn Val Glu Val His
225                 230                 235                 240

ACA GCT CAG ACA CAA ACC CAT AGA GAG GAT TAC AAC AGT ACT CTC CGG      768
Thr Ala Gln Thr Gln Thr His Arg Glu Asp Tyr Asn Ser Thr Leu Arg
                245                 250                 255

GTG GTC AGT GCC CTC CCC ATC CAG CAC CAG GAC TGG ATG AGT GGC AAG      816
Val Val Ser Ala Leu Pro Ile Gln His Gln Asp Trp Met Ser Gly Lys
            260                 265                 270

GCA TTC GCA TGC GCA GTC AAC AAC AAA GAC CTC CCA GCG CCC ATC GAG      864
Ala Phe Ala Cys Ala Val Asn Asn Lys Asp Leu Pro Ala Pro Ile Glu
        275                 280                 285

AGA ACC ATC TCA AAA CCC AAA GGG TCA GTA AGA GCT CCA CAG GTA TAT      912
Arg Thr Ile Ser Lys Pro Lys Gly Ser Val Arg Ala Pro Gln Val Tyr
    290                 295                 300

GTC TTG CCT CCA CCA GAA GAA GAG ATG ACT AAG AAA CAG GTC ACT CTG      960
Val Leu Pro Pro Pro Glu Glu Glu Met Thr Lys Lys Gln Val Thr Leu
305                 310                 315                 320

ACC TGC ATG GTC ACA GAC TTC ATG CCT GAA GAC ATT TAC GTG GAG TGG     1008
Thr Cys Met Val Thr Asp Phe Met Pro Glu Asp Ile Tyr Val Glu Trp
                325                 330                 335

ACC AAC AAC GGG AAA ACA GAG CTA AAC TAC AAG AAC ACT GAA CCA GTC     1056
Thr Asn Asn Gly Lys Thr Glu Leu Asn Tyr Lys Asn Thr Glu Pro Val
            340                 345                 350

CTG GAC TCT GAT GGT TCT TAC TTC ATG TAC AGC AAG CTG AGA GTG GAA     1104
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        355                 360                 365

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    370                 375                 380

GAG GGT CTG CAC AAT CAC CAC ACG ACT AAG AGC TTC TCC CGG ACT CCG     1200
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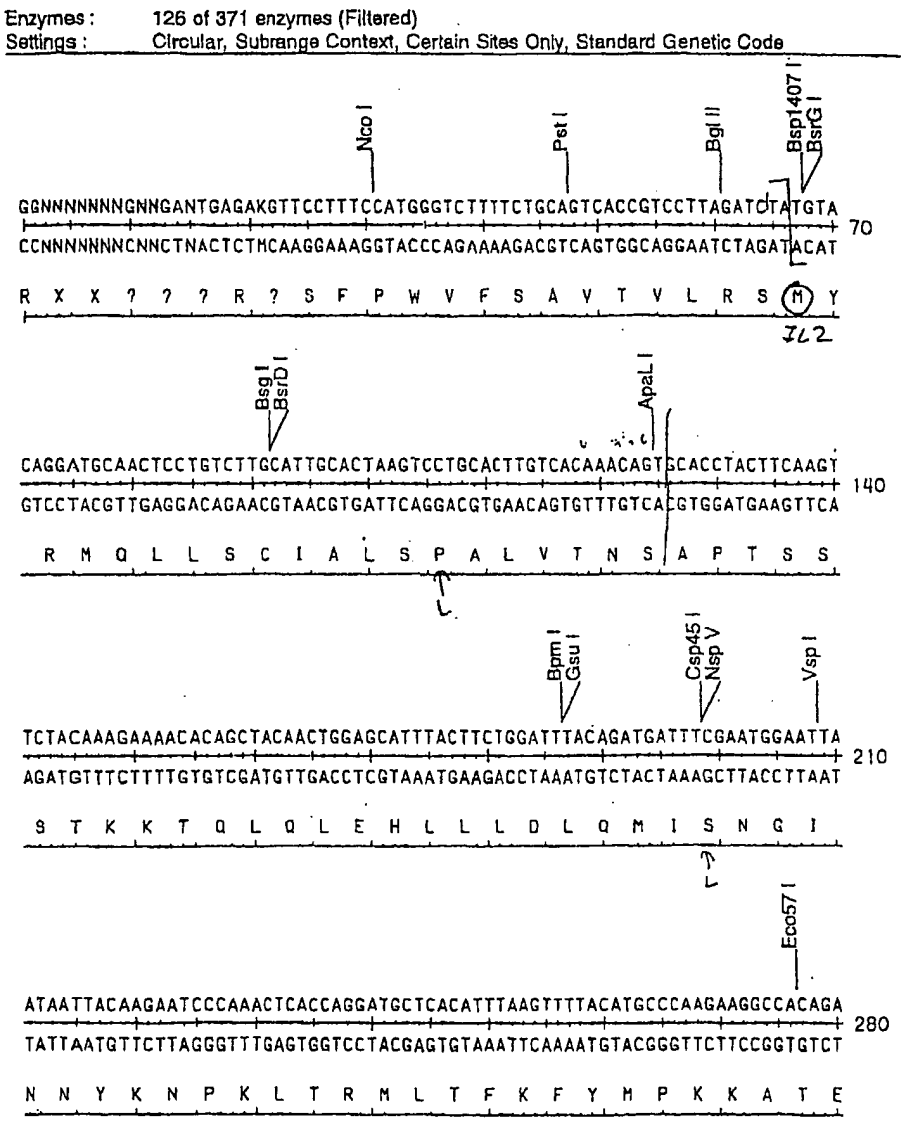
Figure 9B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70
R X X ? ? ? R ? S F P W V F S A V T V L R S M Y
JL2
Bsg I
BsrD I
ApaL I
CAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCCTGCACTTGTCACAAACAGTGCACCTACTTCAAGT
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140
R M Q L L S C I A L S P A L V T N S A P T S S
L
Bpm I
Gsu I
Csp45 I
Nsp V
Vsp I
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S T K K T Q L Q L E H L L L D L Q M I S N G I
Eco57 I
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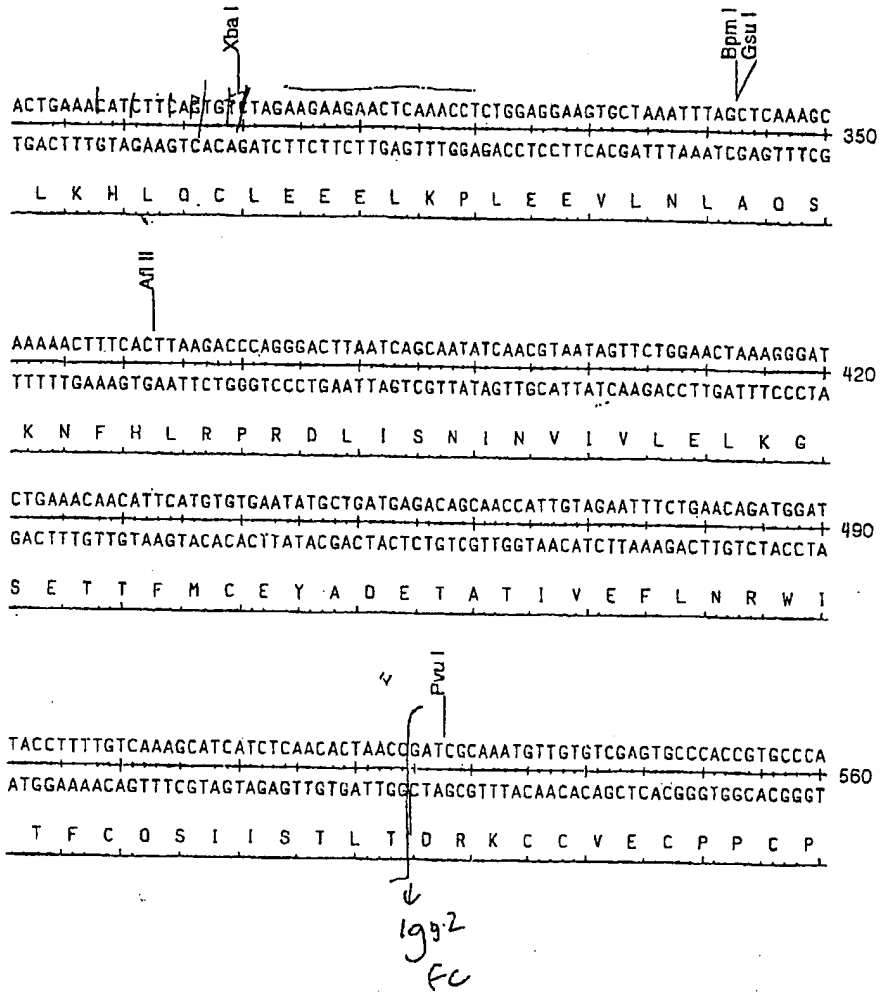
Figure 10A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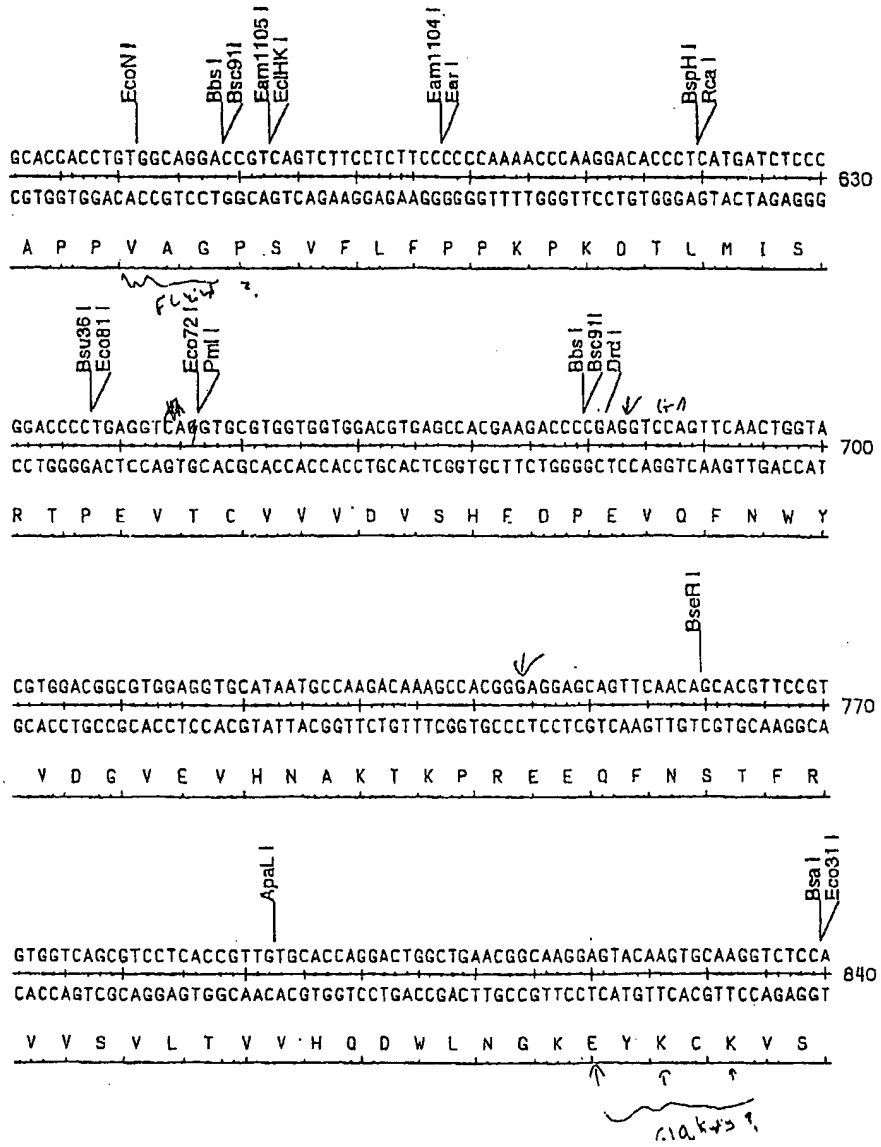
Fc

Figure 10B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Eco81 I
Eco72 I
Pml I
Bbs I
Bsc91I
Drd I
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R T P E V T C V V V D V S H E D P E V Q F N W Y
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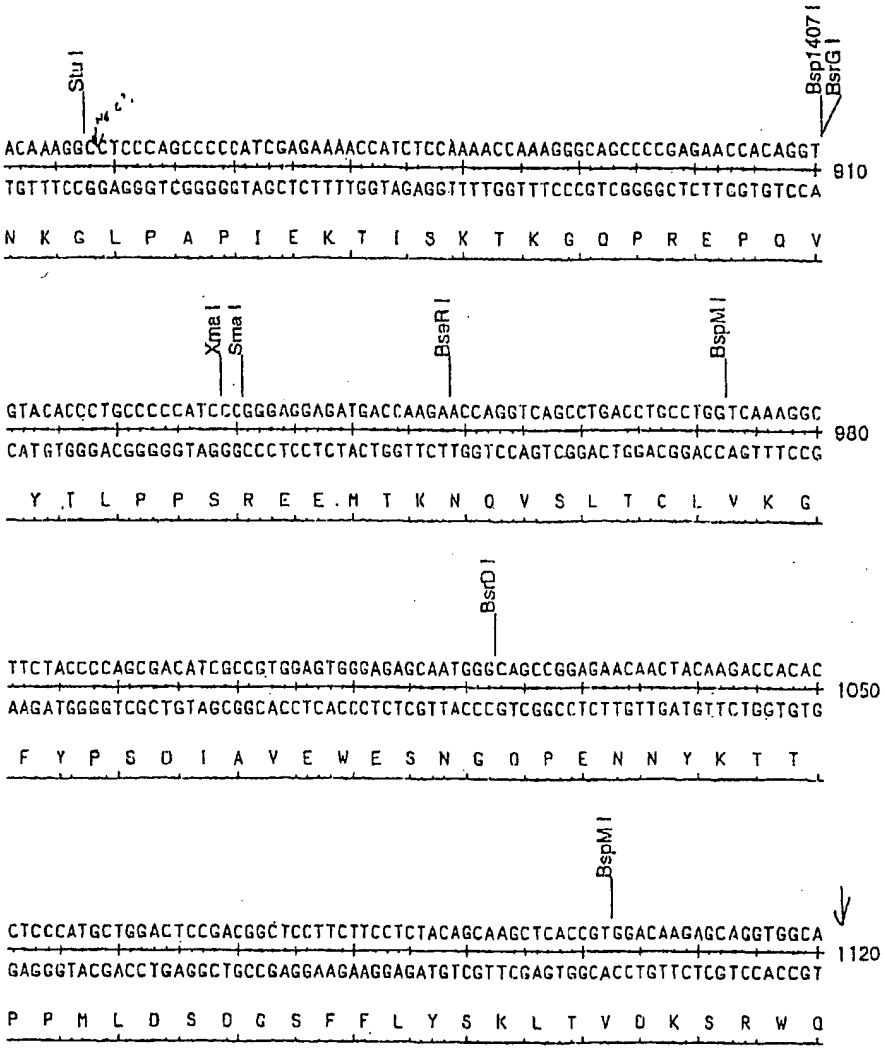
Figure 10C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Sma I
BssR I
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BspM I
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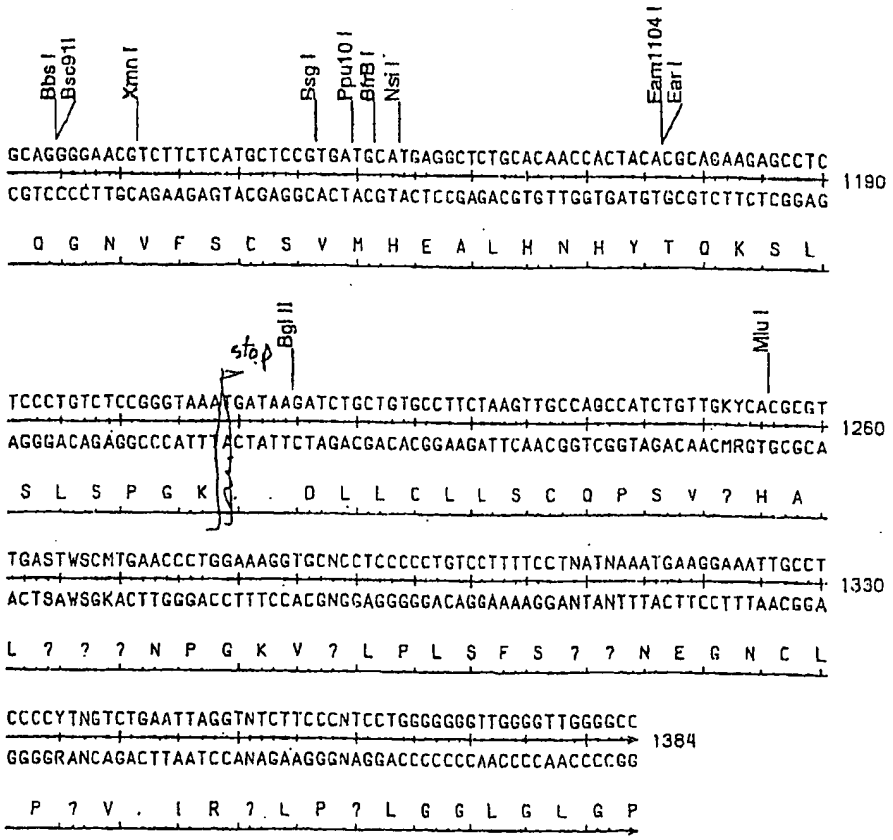
Figure 10D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1384
P ? V . I R ? L P ? L G G L G L G P

Figure 10E

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/00, 9/00, 38/19, 48/00, 38/20, 39/21, C12N 15/19, 15/62, A61K 31/70, C12N 15/48

A3

(11) International Publication Number: **WO 99/16466**

(43) International Publication Date: 8 April 1999 (08.04.99)

(21) International Application Number: PCT/US98/20321

(22) International Filing Date: 29 September 1998 (29.09.98)

(30) Priority Data:

60/060,338	29 September 1997 (29.09.97)	US
08/990,180	12 December 1997 (12.12.97)	US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications

US	08/990,180 (CIP)
Filed on	12 December 1997 (12.12.97)
US	60/060,338 (CIP)
Filed on	29 September 1997 (29.09.97)

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LETVIN, Norman, L. [US/US]; 36 Brackett Road, Newton, MA 02158 (US). BAROUCH, Dan, H. [US/US]; 19 Bowker Street, Brookline, MA 02146 (US).

(74) Agents: HOGLE, Doreen, M. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
3 June 1999 (03.06.99)

(54) Title: VACCINE COMPOSITIONS AND METHODS OF ENHANCING VACCINE EFFICACY

(57) Abstract

The invention provides methods, vaccine compositions and plasmid constructs which enhance the immune response of a vaccine.

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EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 98/20321

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K9/00 A61K38/19 A61K48/00 A61K38/20
A61K39/21 C12N15/19 C12N15/62 A61K31/70 C12N15/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. VIEWEG ET AL.: "Preclinical studies toward gene therapy of prostate cancer" JOURNAL OF CELLULAR BIOCHEMISTRY, SUPPLEMENT, vol. 21A, 1995, page 360 XP002097488 New York, NY, USA see abstract C6-019 --- -/--	1-5,8, 11,12, 15,20-24

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1999	09/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte...onal Application No
PCT/US 98/20321

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	C. LEKUTIS ET AL.: "HIV-1 env DNA vaccine administered to rhesus monkeys elicits MHC class II-restricted CD4+ T helper cells that secrete IFN-gamma and TNF-alpha." THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 9, 1 May 1997, pages 4471-4477, XP002097489 Baltimore, MD, USA cited in the application see abstract see section HIV-1 env DNA vaccination	1-54
A	A. WEINBERG ET AL.: "Recombinant interleukin 2 as an adjuvant for vaccine-induced protection." THE JOURNAL OF IMMUNOLOGY, vol. 140, no. 1, 1 January 1988, pages 294-299, XP002097490 Baltimore, MD, USA cited in the application see the whole document	1-54
A	S. PRAYAGA ET AL.: "Manipulation of HIV-1 gp120-specific immune responses elicited via gene gun-based DNA immunization." VACCINE, vol. 15, no. 12/13, August 1997, pages 1349-1352, XP002097491 Guildford, GB see abstract	1-54
A	N. LETVIN ET AL.: "Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE U.S.A., vol. 94, no. 17, 19 August 1997, pages 9378-9383, XP002097492 Washington, DC, USA see abstract	1-54
P,X	D. BAROUCH ET AL.: "Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration." THE JOURNAL OF IMMUNOLOGY, vol. 161, no. 4, 15 August 1998, pages 1875-1882, XP002097493 Baltimore, MD, USA see the whole document	1-54

1

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 98/20321

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 20, 23 and 26-48 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.